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PRODUCTION OF STEM CELL-DERIVED RETICULOCYTES FOR THE *IN VITRO* CULTURE OF *PLASMODIUM VIVAX* BLOOD CYCLE

Jury:

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Deux intellectuels assis vont moins loin qu'une brute qui marche
…. Mais l'intellectuel, quand il se lèvera, il ira dans la bonne direction !

Michel Audiard, un taxi pour Toubrouk (1961)

Table of content:

ABBREVIATION LIST:

BM: Bone marrow

BMP 4: Bone morphogenetic protein 4

CD: Cluster of differentiation

CDC: Center of disease control

DARC: Duffy antigen chemokine receptor

DMEM: Dulbecco modified Eagle's medium

EPO: Erythropoietin

hESC: Human embryonic stem cell

FACS: Fluorescence assisted cell sorting

FCS: Fetal calf serum

FLT-3: Fms-like tyrosine kinase 3

Hb: Hemoglobin

HDS: Hydrocortisone

HSC: Hematopoietic stem cell

HSPC: Hematopoietic stem/ progenitor cell

IL-3: Interleukin 3

IL-6: Interleukin 6

IMDM: Iscove's Modified Dulbecco's Medium

iMEF : Inactivated mouse embryonic fibroblast

iPSC: Induced pluripotent stem cells

MACS: Magnetic assisted cell sorting

MNC: Mononuclear cells

PB: Peripheral blood

PBS: Phosphate buffered saline

PvDBP: *Plasmodium vivax* duffy binding protein

RBC: Red blood cells

RNA: Ribonucleic acid

RPMI: Roswell park memorial institute (medium)

SCF: Stem cell factor

TPO: Thrombopoietin

UCB: umbilical cord blood

VEGF: Vascular endothelial growth factor

WHO: World health organization

SUMMARY

Research on the malaria parasite *Plasmodium vivax* (*P. vivax*) lags behind the one on *Plasmodium falciparum* (*P. falciparum*). This applies also to the parasite's biology as an *in vitro* culture of the *P. falciparum* blood cycle is already available. Conversely, despite several attempts, a reliable and reproducible *P. vivax in vitro* culture is not currently available. One of the main obstacles is represented by the *P. vivax* preference for invading young erythrocyte, named reticulocytes. The proportion of reticulocytes, among circulating red blood cells, in the adult blood stream is extremely low (0.5-1%) and their lifespan (in the blood stream) is only 1 day. Therefore, it is extremely difficult to obtain from the peripheral blood an adequate amount of reticulocytes able to support a *P. vivax in vitro* culture.

To tackle this problem and been able to provide a sufficient amount of target cells to maintain the parasite cycle, the production of reticulocytes starting from hematopoietic stem cells (HSC), erythrocytes progenitors, has been investigated. HSC are multipotent cells, with properties of self-renewal and erythroid differentiation.

Following a 14-day protocol, reticulocytes could be produced from 3 different sources of HSC, namely cord blood (CB, 18% reticulocytes), bone marrow (BM, 21% reticulocytes) and peripheral blood mononuclear cells (PBMC, 31% reticulocytes). Reticulocytes derived from any of these sources could be invaded by *P. vivax* and some re-invasion could be observed after 3 days. Nevertheless, no real *in vitro* multiplication could be observed.

The possibility to expand the population of HSC following a simple 5 days protocol (10 fold expansion for CB and 3 fold expansion for BM) coupled with the possibility to cryopreserve reticulocytes produced allow the creation of a stock of reticulocytes ready to be used for a continuous culture.

We also investigated the influence of hemoglobin in the development of *P. falciparum* and *P. vivax.* After 5 days, *P. falciparum* continuous synchronous culture was observed with fetal hemoglobin (HbF) while asynchronization occurred with adult hemoglobin (HbA). *P. vivax* invasion rates did not differ according to the type of hemoglobin present in culture.

In conclusion, *P. vivax* could invade reticulocytes produced in vitro from HSPC, regardless of their source. The possibility of expanding the precursor cells and of cryopreserving the reticulocytes produced allows the creation of adequate stocks of target cells that could maintain a continuous culture. Nevertheless, it was no possible to achieve the ultimate goal of a continuous *P. vivax* culture, though such failure cannot be explained by reticulocyte quality or the type of hemoglobin. Some elements/factors, currently unknown, are probably essential for the maintenance of the culture. Metabolomics studies comparing *in vivo* and *in vitro* infections may be able to identify the missing elements necessary for the *in vitro* growth of *P. vivax*.

SAMENVATTING

Onderzoek naar de malariaparasiet *Plasmodium vivax* (*P. vivax*) loopt achter op dat naar *Plasmodium falciparum* (*P. falciparum*). Dit geldt zeker ook voor de biologie van de parasiet aangezien er, ondanks verschillende pogingen, enkel een *in vitro* cultuursysteem van de *P. Falciparum* bloedcyclus beschikbaar is, en geen betrouwbare en reproduceerbare in vitro kweek van *P vivax* mogelijk is. Een van de belangrijkste obstakels voor de in vitro expansie van *P vivax* is de voorkeur van de parasiet voor het binnenvallen van jonge erytrocyten , genaamd reticulocyten . Het percentage reticulocyten onder circulerende rode bloedcellen in de volwassen bloedstroom is extreem laag (0,5-1 %) en hun levensduur (in het bloed) is slechts 1 dag . Daarom is het uiterst moeilijk om de hoeveelheden reticulocyten die nodig zijn om P. vivax *in vitro* te ondersteunen te verkrijgen uit bloed.

Om de parasietcyclus te voorzien van een voldoende hoeveelheid targetcellen werd de generatie van reticulocyten canuit hemapoietische stamcellen (HSC), voorlopers van de errythrocyten, onderzocht. HSC zijn multipotente cellen met zowel de capaciteit to zelf vernieuwing als die tot erytrocyt differentiatie.

Met behulp van een protocol van 14 dagen konden we reticulocyten genereren vanuit 3 verschillende bronnen van HSC, namelijk navelstrengbloed (CD, 18% reticulocyten), beenmerg (BM, 21% reticulocyten) en perifere mononucleaire bloedcellen (PBMC, 31% reticulocyten). Er kon invasie worden aangetoond in reticulocyten gegenereerd van elk van de drie bronnen maar, ondanks de observatie van enkele herinvasies na drie dagen, werd er geen echte *in vitro* multiplicatie waargenomen.

De mogelijkheid om de HSC populatie te expanderen gebruik makend van een simpel 5 dagen durend protocol (10-voudige expansie voor CB en 3-voudige expansie voor BM), gekoppeld met de mogelijkheid om reticulocyten in te vriezen, liet wel toe een reticulocytenstock aan te maken voor gebruik in een continue cultuur.

Verder hebben we ook de invloed van hemoglobine onderzocht op de ontwikkeling van *P. Falciparum, P. Vivax* en een continue, synchrone cultuur van *P. Falciparum* met foetale hemoglobine (HbF). Dit terwijl

desynchronisatie reeds optrad na 5 dagen wanneer gebruik werd gemaakt van adulte hemoglobine(HbA). Voor *P. Vivax* bleek de invasie-efficientie niet afhankelijk van het gebruikte hemoglobinetype.

Om samen te vatten hebben we aangetoond dat invasie mogelijk is van *in vitro* van HSC geproduceerde reticulocyten, en dit onafhankelijk van hun bron. De mogelijkheid om de precursorcellen te expanderen en de reticulocyten te cryopreserveren liet ons toe voldoende grote stocks aan te maken om een continue cultuur te onderhouden. Ondanks dit was het niet mogelijk om het uiteindelijke doel, een continue in vitro cultuur van P vivax, te bekomen. Dit kon echter niet worden verklaard door de kwaliteit van gegenereerde reticulocyten of door het type hemaglobine. Waarschijnlijk zijn sommige nog ongekende elementen essentieel voor het onderhouden van de cultuur. Een studie waarin het metaboloom wordt bestudeerd tijdens *in vitro* en *in vivo* infecties zou de onberekende elementen die nodig zijn voor een in vitro groei van de parasiet P vivax kunnen identificeren.

INTRODUCTION:

Chapter I: Malaria

Numbers and facts

Malaria is a parasitic disease due to a protozoa of the order of haemosporida genus *Plasmodium* [1]. More than 200 *Plasmodium* species can infect different hosts (avian, monkey, mouse…) though only five of them, *P. falciparum*, *P. vivax*, *P. ovale* (*P. ovale curtisi* and *P. wallikeri), P. malariae and P. kwnolesi*, can infect humans. In 2010, it was estimated there were about 219 million malaria cases and nearly 660 000 malaria-related deaths (WHO report 2012). Artemisinin-based combination therapies (ACTs) are the currently recommended first line treatment for uncomplicated *P. falciparum* malaria (WHO, 2012). For severe malaria, regardless of the parasite species, intravenous artesunate or quinine, when the former is not available, are recommended. *P. vivax* uncomplicated malaria may still be treated with chloroquine and primaquine, the latter to clear the liver stages of the parasite. Most antimalarial drugs target schizonts by blocking the detoxification of the heme (during hemoglobin digestion), which is toxic for the parasite [3]. However, parasites can develop resistance to treatment; for example P. falciparum parasites with particular mutations in the *Plasmodium falciparum* Chloroquine Resistance transporter gene (pfCRT) are able to reduce the concentration of chloroquine inside their digestive vacuole and hence escape to the drug activity [4].

Life cycle (fig.1)

During a blood meal on a malaria-infected person, mosquitoes may ingest also gametocytes present in the blood. If this occurs, a male and female gametocyte fuse to give rise to an ookinete that migrates through the mosquito's midgut where it further develops into an oocyst. The latter, at the end of its development, will release sporozoites that migrate to the mosquito's salivary glands, to be injected in the human host at the next blood meal. The cycle in the vector is known as the sexual cycle of the parasite.

When released into the blood stream, at the time of a mosquito bite, sporozoites migrate rapidly to the liver where they will invade hepatocytes. Here they will initiate a phase of

multiplication resulting in the formation of an exo-erythrocytic schizont that will release merozoites in the bloodstream. Within seconds, they will adhere and invade red blood cells (RBC), where the parasite will develop into different stages (fig.2). The ring stage is characterized by the presence of large chromatin dots and a cytoplasm with a ring-form. This is followed by the trophozoite stage, with an amoeboid shape and pigment dots, known as hemozoin, which are the result of the hemoglobin digestion. Finally, the schizont stage will mature and produce a new generation of merozoites to be released in the blood stream and invade new RBC. The parasite reproduces asexually during the exo-erythrocytic and erythrocytic cycles in the human host.

Following a stress signal (parasite density, anemia, immune response), malaria parasites will also produce gametocytes (microgametocyte male and macrogametocyte female) that will continue the sexual cycle in the vector.

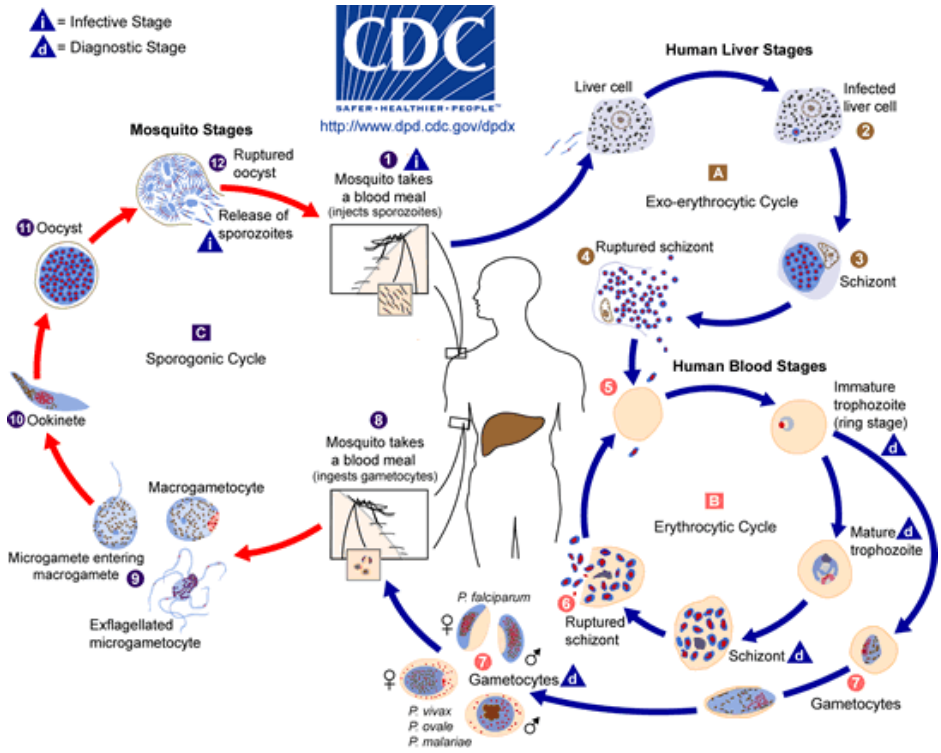

Fig 1: Life cycle of malaria Parasite (source: CDC)

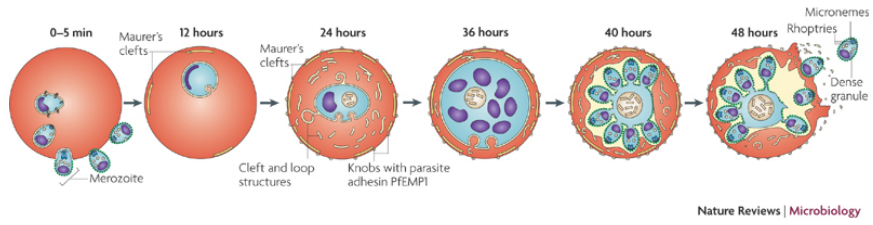

Fig.2: Different stages of *Plasmodium* blood cycle (Maier *et al.*, Nature Rev. Microbiol 2009)

Malaria epidemiology

Malaria is a tropical disease that is located mainly in sub-Saharan Africa, the Amazonian region and South-east Asia (fig 3). Malaria transmission can be perennial and occur the whole year or seasonal, during a few months, mostly coinciding with rainfalls. It can also be stable, when it does not vary substantially between years, even if it is seasonal, or unstable, with marked variation between years [5].

P. vivax transmission occurs mainly in South-east Asia and South America while it is almost absent in Africa (fig.4). This is related to the distribution of the Duffy receptor in the human population, a receptor found on the surface of the RBC (fig.5). Indeed, the human population in sub-Saharan Africa is mainly Duffy negative and this could explain the paucity of *P. vivax* malaria as the Duffy receptor is important for the invasion of RBC.

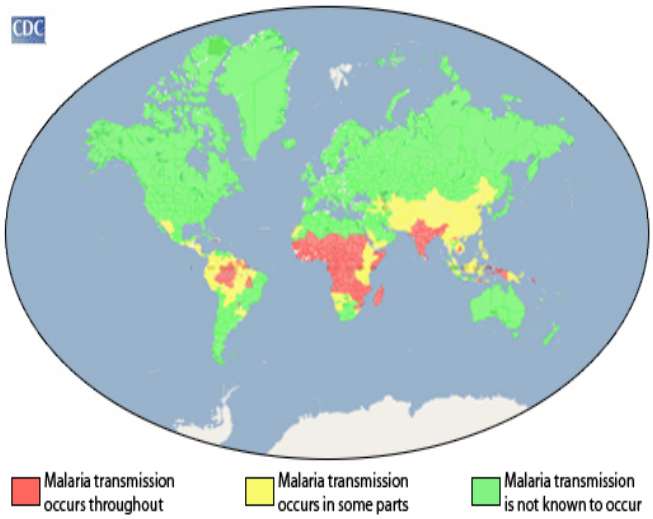

Fig 3: Map of the malaria global distribution (source: CDC)

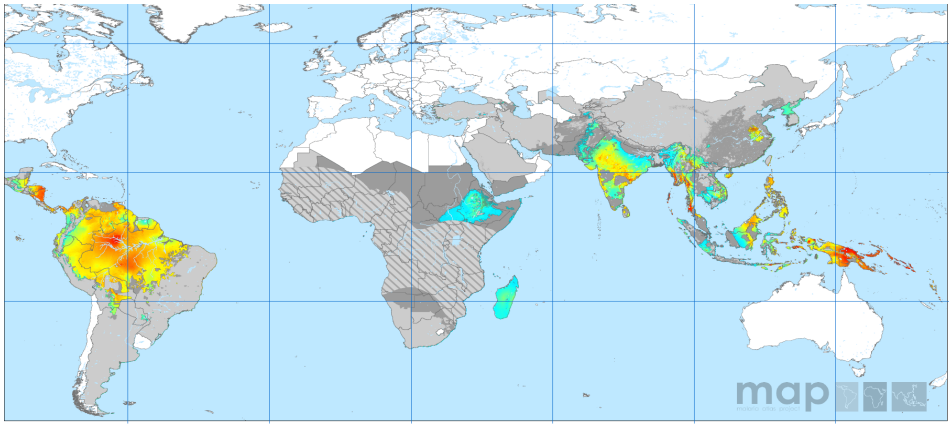

Fig 4. : Map of Plasmodium vivax distribution in 2010.

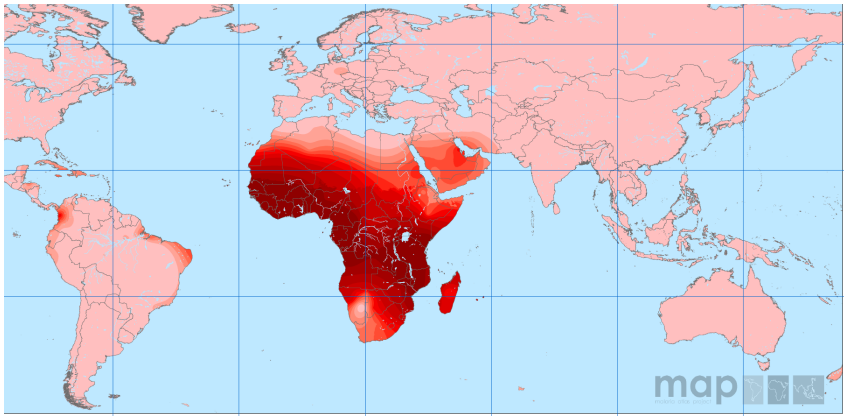

Fig 5. : Map of the duffy Negative-population distribution

Chapter II: *Plasmodium vivax*

Characteristics

P. vivax has some specific features. One of the most important is its capacity to produce dormant forms (hypnozoites) in the human liver [6]: once entered in the liver under a sporozoite form, *P. vivax* can either develop into schizont and release merozoites in the blood stream or stop its development and remain dormant until an unknown signal activates it to continue its development into schizont and then merozoites to be released in the blood stream, resulting in a relapse [7].

Another specific trait of *P. vivax* is its preference to invade young erythrocytes (named reticulocytes) [8] using the Duffy receptor (DARC) on reticulocyte's surface and the PvDBP (*Plasmodium vivax* Duffy Binding Protein) [9]. Such exclusive interaction with the DARC has been recently questioned as there have been reports of *P. vivax* cases in Duffy negative individuals from Sub-Saharan Africa and the Amazonian region, suggesting that other unidentified parasite's and reticulocyte's receptors are involved in the invasion process [10].

Morphologically, *P. vivax* can be discriminated from other plasmodium species by identifying the presence of Shuffner's dots (brick-red dots) in the cytoplasm of the infected reticulocyte. The infected reticulocyte is also larger than a *P. falciparum* infection and the *P. vivax* trophozoite has an "amoeboid" shape.

Plasmodium vivax in vitro culture

Review

Cell PRESS

1912–2012: a century of research on *Plasmodium vivax in vitro* culture

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Keywords: Malaria, *Plasmodium vivax*, *in vitro* culture, reticulocyte

Abstract

The development of a continuous *Plasmodium vivax* blood cycle *in vitro* was first attempted one hundred years ago. Since then, and despite the use of different methods, only short-term cultures have been achieved so far. The available literature has been reviewed in order to provide a critical overview of the currently available knowledge on *P. vivax* blood cycle culture systems and identify some unexplored ways forward. Results show that data accumulated over the past century remain fragmented and often contradictory, making it difficult to draw conclusions. There is the need for an international consortium on *P. vivax* culture able to collect, update and share new evidence, including negative results, and thus

better coordinate current efforts towards the establishment of a continuous *P. vivax* culture.

The need for an *in vitro* model of *Plasmodium vivax.*

The lack of a reliable *in vitro* model of the *P. vivax* blood cycle is probably a major reason for the currently existing knowledge gaps on the biology of this parasite [11]. Research on *P. vivax* has lagged behind that on *Plasmodium falciparum* despite the fact that it is the most widely distributed human *Plasmodium* species outside Sub-Saharan Africa [12]. The focus on *P. falciparum* was justified by its burden and the emergence of resistance to commonly used treatments [13]. Moreover, the availability of a reliable *in vitro* culture system for *P. falciparum* [14] has allowed for an in-depth understanding of its biology and pathophysiology. Recently, *P. vivax* has been put at the forefront of the malaria research agenda following the recent shift from malaria control to elimination and eradication [15], which calls for an enhanced knowledge on the biology of human malaria parasites. In addition, the emergence of *P. vivax* chloroquine resistance [16] has contributed to the increased interest in the research on this parasite. The development of an *in vitro* culture system for *P. vivax* has been clearly identified as a research priority by the Malaria Eradication Research Agenda (malERA) initiative [17].

The first attempt to culture *P. vivax* was published one hundred years ago by Bass *et al*. [18]. Since then, other research groups have attempted to set up a reliable and continuous culture, but results remain inconclusive and not readily reproducible. This review summarizes a hundred years of research on this topic and critically reviews the methods employed and the results obtained, with the aim of identifying outstanding questions and potential solutions.

A systematic review of the literature (published and unpublished) was done using PubMed and the Internet (Google, Google Scholar, and conference reports) with the following key words: '*P. vivax*' '*in vitro* culture', and 'blood cycle'. Moreover, reference lists of all articles were screened manually. No date or language limits were included in the search.

***P. vivax* life cycle stages and *in vitro* cultures .**

The life cycle of *P. vivax,* as for other human parasites, comprises an exogenous sexual phase (sporogony) in the Anopheline vector and an endogenous asexual phase (schizogony) with multiplication in the human host. The schizogony includes the parasite's development in the liver (exo-erythrocytic schizogony) and in red blood cells (RBCs) (erythrocytic schizogony). There have been several attempts, with varying degrees of success, to produce *in vitro* cultures of the different development stages of *P. vivax* life cycle. Indeed, *P. vivax* ookinetes, resulting from the parasite sexual reproduction in the mosquito's gut, have been produced using infected human blood incubated with a gametogenesis solution, with the resulting zygotes cultivated in ookinete medium. Ookinetes were produced in most (86%) of the

optimized *in vitro* cultures [19]. Nevertheless, an *in vitro* model of the *Anopheles* midgut cells for the culture of *P. vivax* oocysts is not available yet.

During the exo-erythrocytic schizogony, *P. vivax* produces dormant forms (hypnozoites) in liver cells that are responsible for relapses occurring several months or years after the primary infection [20]. Therefore, understanding the biology of this specific stage is crucial as it contributes to maintain the human parasite reservoir in endemic areas and challenges *P. vivax* elimination. The main bottleneck for the liver stage culture is the establishment of a functional hepatocyte cell line. Merozoites able to invade RBCs were produced by infecting a human hepatocyte cell line (HC-04), with *P. vivax* sporozoites, however, the percentage of infected hepatocytes was extremely low (0.066%) [21]. New advances in the production of functional hepatocytes (glycogen storage, cytochrome activity, low-density lipoprotein uptake, etc.) derived from stem cells [22, 23] may overcome this problem.

P. vivax erythrocytic stage _in vitro_ culture:

P. vivax blood stage *in vitro* assays and culture can be divided into four categories, namely: (i) single schizogony cycles, (ii) invasion assays, (iii) short-term, and (iv) long-term cultures.

Single schizogony cycles (SSC)

SSC consist of a single maturation cycle from ring to schizont stage without new invasions; parasites collected from infected patients are maintained in culture for up to 48 hours without adding new RBCs. This type of culture is mainly used to determine the influence of different medium components on the intra-erythrocyte parasite development [24] or the parasite's sensitivity profile to different anti-malarial drugs [25-27]. The latter have been established using either (^{3}H) hypoxanthine [26] or by counting the number of rings matured into schizonts on a Giemsa stained blood smear [27]. As reported earlier [25, 26], this technique is limited to drugs acting on early asexual blood stages, and results are highly variable, though Russell *et al.* [27] recently reported on a reproducible *in vitro* test for drug sensitivity (Figure 1).

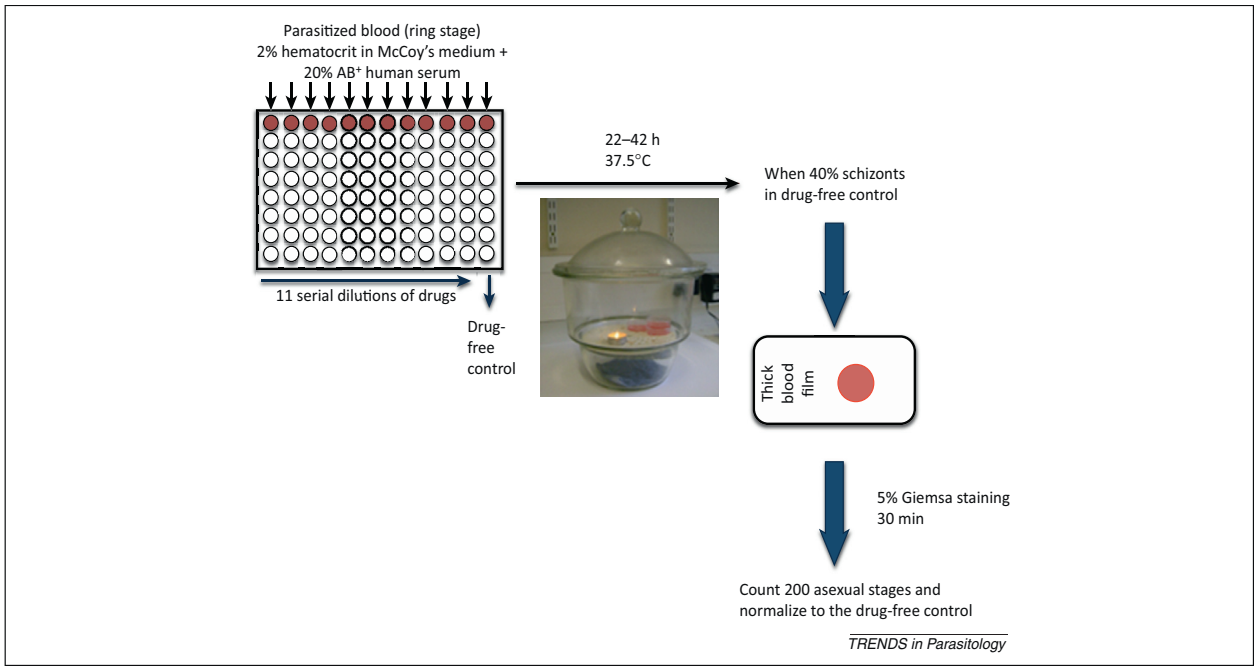

Figure 1. Scheme of the *Plasmodium vivax* drug test protocol. *P. vivax*-infected blood (from patient) containing a majority of ring forms is distributed in a 96-well plate containing 11 serial dilutions of the drug to test. One control well is kept free of drug and will be used for monitoring parasite development. The plate is placed in a candle jar at 37°C for 22–42 h, and the experiment is stopped when at least 40% of schizonts are found in the control well. A thick smear is made from each of the 11 wells and the number of schizonts per 200 asexual stage parasites is determined for each drug concentration and normalized to the control well. The IC_{50} corresponds to the drug concentration required to obtain 50% of inhibition of the schizont maturation.

Invasion assays:

Invasion assays (Table 1) consist of a first maturation step, as for the SSC, followed by a concentration step of the infected RBCs and an additional 24h culture. Parasites are concentrated on a Percoll gradient to obtain over 90% infected RBCs. The latter are then mixed with new reticulocytes and cultured (RPMI or McCoy medium) for an additional 24h to allow new invasions (Figure 2). These assays are essential to study the role of specific receptors involved in the erythrocyte invasion, e.g., *Pv* Duffy Binding Protein (*Pv*DBP, parasite surface) and the Duffy receptor (RBC surface) [28-30]. Moreover, invasion assays can also be used to test the susceptibility of reticulocytes from different sources to *P. vivax* invasion [31, 32]. Nevertheless, the small amount of infected RBCs obtained after Percoll centrifugation allows only a few experiments. Among the six published invasion assays, parasite densities ranged from 0.1% and 22.3%, the lowest value being observed in a protocol without concentration step (Table 1).

Table 1. List of publications and methods used in *Plasmodium vivax* invasion assays

Date	*P. vivax* source	*P. vivax* concentration	Reticulocyte concentration	Medium	Maximal parasite density post-invasion
1987	Belem strain from *Saimiri* monkey	Concentration on 54% Percoll	Concentration on 65% Percoll	Not provided	Not provided
1988	Palo Alto strain from *Aotus nancymai*	Concentration on 58% nicodenz	Monkey blood after artificially induced anemia	RPM1 1640 with HEPES, $NaHCO_3$, neomycin + 20% serum	11.9%
1989	Belem strain from *Saimiri* monkey	Concentration on 54% Percoll	Concentration on 65% Percoll	RPMI 1640 with HEPES, hypoxanthine, D-glucose + 15% serum	15.0%
2007	Infected patients	No concentration	No concentration	McCoy's 5A medium with HEPES, $NaHCO_3$, L-glutamine, gentamicin + 25% serum	0.1%
2011	Infected patients	Concentration on 45% Percoll	Concentration on 60% Percoll	McCoy's 5A medium with L-glutamine, gentamicin, glucose + 20% serum	22.3%
2012	Infected patients (cryopreserved isolates)	Concentration on 45% Percoll	HSC-derived reticulocytes	McCoy's 5A medium with L-glutamine, glucose + 20% serum	2.5%

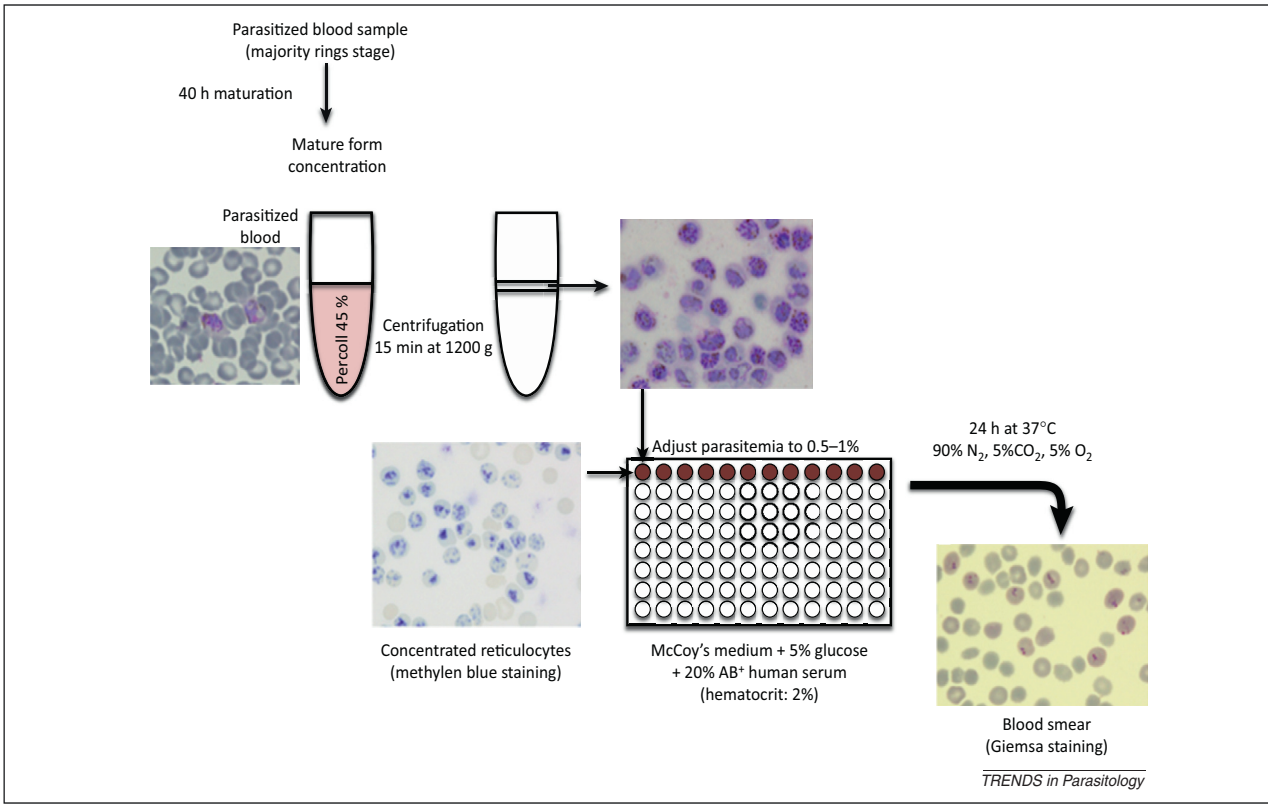

Figure 2. Scheme of the *Plasmodium vivax* invasion assay protocol. *P. vivax*-infected blood containing a majority of ring forms is kept *in vitro* for maturation until schizont stage. The mature forms are concentrated on 45% Percoll to obtain a population of more than 90% parasites. The latter are then mixed with reticulocyte-enric blood for a final 0.5–1% parasitemia in a 96-well plate. The plate is kept at 37°C for 24 h in a chamber filled with a gas mixture (90% N_2–5% CO_2–5% O_2). At the end of experiment, a blood smear is done and parasites counted after Giemsa staining.

Short-term cultures

By definition, these *in vitro* cultures are maintained for less than a month and, since they include several schizogony cycles, they offer the possibility to study the parasite development in RBCs (Table 2). In 1912, *Bass et al.* published the first report on a short-term culture of *P. vivax* (collected from patients) that could be maintained for up to four

schizogony cycles [18]. Subsequently, in most published work the parasite density was reported to increase during the first 96 hours and then decline [33-36], though in some experiments the decline was observed from the very beginning of the culture [37-39]. Golenda and colleagues were the only ones that reported a doubling of the parasite population at each cycle by using a *P. vivax* Chesson strain adapted in monkeys [40]. This was then cultured in M5AMM medium regularly supplemented with blood from hematochromatosis patients enriched in reticulocytes using centrifugation on homologous plasma. However, this protocol has never been successfully implemented by other research groups.

A preliminary parasite concentration step, before starting the culture, was done in only one of the nine published studies, but did not result in better parasite growth [39]. All other short-term cultures were initiated with the blood of infected monkeys or patients, to which reticulocytes were regularly added. None of these studies investigated the parasite's biology, as the main objective was the optimisation of the culture conditions, for example, medium [34] and gas composition [36].

Table 2. List of publications and methods used in *Plasmodium vivax* short-term *in vitro* blood cycle

Date	*P. vivax* source	Reticulocyte source	Medium	Other conditions: support[a], temperature, gas[b]	Days in culture	Parasite growth
1912	Infected patients	Human erythrocytes	Locke's fluid w/o calcium chloride + inactivated human serum + ascitic fluid + dextrose	Culture tube, temperature: 40°C gas: NA	4 cycles	No indications given
1979	Vietnam–Palo Alto strains from *Aotus* monkey + infected patient (Philippines)	Monkey red blood cell fraction I	RPMI 1640	Perfusion-jar, temperature: NA, gas: NA	8 days	Increased until day 5 and then decreased
1985	Infected patients (Thailand)	Human red blood cells	RPMI 1640 or Waymouth 752/1 or SCMI 612 + amino acid mixture + hypoxanthine + vitamin solution + 15% AB^+ human serum	Culture dishes, temperature 37°C, gas: candle jar	6 days	Low parasite density, poor reinvasion
1991	Infected patients (India)	Erythrocyte suspension	RPMI 1640 + 10% human serum + peptone + glucose + liver extract	Petri dish, temperature: NA, gas: NA	15 days	Good growth in the presence of liver extract
1991	Infected patients (China)	Human erythrocytes	RPMI 1640 + adenine + hypoxanthine + L-glutamine + glucose + ascorbic acid + $MgCl_2$ + $NaHCO_3$ + 15–20% AB^+ serum	Support: NA, temperature: 37°C, gas: candle jar[c]	96 h	Parasite density doubling after 96 h
1992	Chesson strain in *Saimiri* monkey	Human blood enriched in reticulocytes by Percoll gradient centrifugation	RPMI 1640 + $NaHCO_3$ + nutritional supplements + 15% AB serum (heat inactivated)	Flow vessel, temperature: 37°C, gas mixture 1[d]	22 days	Parasite density decreased from the beginning
1997	Chesson strain in *Aotus* monkey	Hemochromatosis blood enriched in reticulocyte by homologous plasma centrifugation	M5AMM medium + 20% AB^+ human serum	Culture flask, temperature: 37°C, gas mixture 2[e], shaking (100 cpm)[f]	15 days	Parasite density remained stable (parasite population doubling every cycle)
2001	Infected patients (Thailand)	None	RPMI 1640 + HEPES + $NaHCO_3$ + gentamicin + D-glucose + $MgSO_4$ + KH_2PO_4 + $CaCl_2$ + hypoxanthine + ascorbic acid + 50% AB^+ serum	Petri dish, temperature: 37°C, gas: 5% CO_2	10 days	Parasitemia readily decreased (no reticulocytes added)
2012	Infected patients (Thailand)	Cord blood enriched in reticulocytes by centrifugation on Percoll layer	McCoy's medium + glucose + 25% AB^+ human serum	96-well plate, temperature: 37°C, gas mixture[e], pRBC concentration[f,g]	10 days	Low parasitemia

[a]Support: recipient where the *in vitro* culture is done.

[b]Gas: gas environment provided to the parasite culture.

[c]Candle jar = 79% N_2 + 3% CO_2 + 18% O_2.

[d]Gas mixture 1 = 92% N_2 + 3% CO_2 + 5% O_2.

[e]Gas mixture 2 = 90% N_2 + 5% CO_2 + 5% O_2.

[f]The culture was shaken at 100 cpm for 10–12 h during each end of schizogony stage.

[g]The pRBCs were concentrated by Percoll centrifugation to obtain a population of over 90% parasites.

Long-term cultures:

These cultures are maintained for more than one month and aim at obtaining *P. vivax* laboratory adapted strains. Between 1987 and 2007 six long-term cultures were published (Table 3), most of them deriving from the Tragger and Jensen protocol for *P. falciparum.* [14]. Most of them were maintained for 2-3 months, and only one lasted up to 5 months. Though the possibility of re-starting a culture from frozen cultured parasites was published in 1981 [41], this result was never reproduced. Similarly, the results of the protocol able to successfully maintain a *P. vivax* culture for up to 125 days [42] (without any details on actual parasite growth) could not be reproduced. Most publications did not report any *in vitro* parasite multiplication; actually, parasite densities remained extremely low throughout the experiments.

Table 3. List of publications and methods used in *Plasmodium vivax* long-term *in vitro* blood cycle

Date	*P. vivax* source	Reticulocyte source	Medium	Other conditions: support[a], temperature, gas[b]	Days in culture	Parasite growth
1981	Infected patient (Brazil)	Human RBCs	RPMI 1640 + human serum + glucose	Petri dish, temperature: 37°C, gas: CO_2 enriched atmosphere pH acid[c]	43 days	Low parasite density: 2.5‰; no indications on parasite shape
1983	Infected patients (India)	Human RBCs	RPMI 1640 + HEPES + $NaHCO_3$ + 10% AB serum	Petri dish, temperature: 37°C, gas: candle jar[d]	125 days	Parasite density increased, no indication on shape
1995	Infected patient (Turkey)	Human RBCs + anemic patient	RPMI 1640 + HEPES + $NaHCO_3$ + 10% AB serum	Support: NA, temperature: 37°C, gas: candle jar[d]	63 days	Low parasite density
2000	Infected patients (1% parasitemia)	Cord blood	RPMI + 15% human serum A^+ erythrocyte + hypoxanthine (5 mg%) + glutathione (60 mg%) + glucose (500 mg%) + p-aminobenzoic acid (15 mg%)	Support: NA, temperature:37°C, gas: candle jar[d]	52 days	Healthy for 2–3 cycles, then unhealthy with low parasitemia
2007	Infected patients (0.02% to 0.12% parasitemia)	Cord blood + hemochromatosis blood	RPMI 1640 + HEPES 25% AB serum (inactivated) + L-glutamine + hypoxanthine + + gentamycin + $NaHCO_3$	75 cm^2 flask, temperature: 37°C, gas mixture[e] or candle jar[d]	40 days	Low parasitemia, no linear growth
2007	Infected patients (0.001% to 0.1% parasitemia)	Derived from hematopoietic stem cells	McCoy's medium + 25% human serum	12-well plate, temperature: 37°C, gas: 5% CO_2	85 days	Very low parasitemia (0.0015%)

[a]Support: recipient where the *in vitro* culture is done.

[b]Gas: gas environment provided to the parasite culture.

[c]Medium was prepared with a pH acid for the first 6 days and was changed three times a day.

[d]Candle jar = 79% N_2 + 3% CO_2 + 18% O_2.

[e]Gas mixture = 90% N_2 + 5% CO_2 + 5% O_2.

The key players

Sources of P. vivax

Non-human primates are used as *in vivo* models for *P. vivax* cultures, as this parasite can infect monkeys. The Chesson strain of *P. vivax* was first adapted to non-human primates in 1969 [43] and was soon followed by the several other *P. vivax* strains (Table 4). Comparing human and non-human models with the Chesson strain, Collins *et al.* [44] concluded there was no difference in terms of maximum parasite densities obtained, though in non-human models the time to reach the same density was longer (18.9 versus 8.5 days). Monkey-adapted *P. vivax* strains have been used to start *in vitro* cultures [37, 40] and are available online at the Malaria Research and Reference Reagent Resource Centre (MR4; www.mr4.org).

Table 4. *Plasmodium vivax* strains adapted in monkey hosts

P. vivax strains	Monkey hosts
NICA	*Aotus trivirgatus*
ONG	*A. trivirgatus griseimembra*
Panama	*A. trivirgatus*
Thai III	*A. azare bolivensis, A. nancymai, A. lemurinus bolivensis, Saimiri sciureus bolivensis*
India VII	*A. trivirgatus, A. azare bolivensis, A. nancymai, A. lemurinus bolivensis, S. sciureus bolivensis, Pan troglodytes*
Indonesia XIX	*A. lemurinus griseimembra, A. vociferans, A. nancymai S. boliviensis*
Brazil I	*A. vociferans, S. bolivensis*
Chesson	*A. lemurinus griseimembra, A. vociferans, A. nancymai, A. azare bolivensis*
NAM	*A. vociferans*
Apastepeque	*A. trivirgatus*
Salvador I	*A. lemurinus griseimembra, A. azare bolivensis, A. nancymai, S. sciureus bolivensis, Pan troglodytes*

The need of fresh blood samples from infected patients and the usually low parasite densities in *P. vivax* infections have been major obstacles for initiating *in vitro* cultures. Though freshly infected blood can be immediately used for culture [45-47], a prior Percoll centrifugation, as done by Borlon *et al.* [39], could partly solve the problem of low parasite density. However, this procedure has not been implemented in long-term cultures yet. In addition, the recent development of a cryopreservation protocol for human *P. vivax* isolates [48] that enables acquisition of isolates with similar invasion efficiency as fresh isolates [39] could represent, if reproducible, an important development towards a continuous *in vitro* culture.

The *in vitro* adaptability of *P. vivax* isolates directly from human patients is extremely variable and depends on the initial density as well as on other inherent characteristics of the parasite. Indeed, according to Lu [49], 60% of isolates with a density ≥0.1% could be successfully maintained *in vitro* for 42 hours while only 25% of those with a density <0.02% could achieve the same result.

Sources of reticulocytes:

Reticulocytes are the target cells for *P. vivax* during the erythrocytic schizogony [50]. Though whole human blood has been previously used as source of reticulocytes [41, 45], their relatively low concentration (1% of total RBCs) and short lifespan (24h) in the blood circulation makes it unsuitable for *P. vivax* culture. Therefore, several methods for a reliable and continuous source of reticulocytes have been developed. One of them uses blood from hemochromatosis patients who have relatively higher reticulocyte concentrations due to regular therapeutic phlebotomies [40]. Reticulocyte concentration can be further increased to 15-20% by two ultracentrifugation cycles in autologous plasma. Although technically simple, this method requires continuous access to hemochromatosis patients, which also implies inter-individual variability of reticulocytes.

Cord blood contains a higher percentage of reticulocytes (4-5%) than peripheral blood and has been used, even without concentration step, for short-term *P. vivax* cultures [46, 47]. Reticulocyte concentration can be increased up to 58% by centrifugation on a 70% isotonic Percoll [29, 51], or up to 70% by lysing red blood cells with a hypotonic lysing solution [52]; though the latter technique is appropriate only for cord blood and not for adult blood.

A third source of reticulocytes is the production of erythroid cells from stem cells, either hematopoietic (HSC) or embryonic (ESC). Reticulocytes produced from HSCs have been shown to be suitable target cells for *P. vivax* invasion [53, 54] and may represent a major step towards the continuous culture. A differentiation protocol, on mouse stromal cells (MS5), of HSCs (isolated from umbilical cord blood) into fully mature human RBCs was first published in 2005 [55]. Using a modified protocol without stromal cells, Panichakul *et al*. [54] obtained 0.5% of reticulocytes after 14 days of maturation, while Noulin *et al*. [53] reported yields up to 20% of reticulocytes. In addition, the latter could be cryopreserved and successfully invaded by *P. vivax* after thawing.

Culture conditions

Culture conditions can be divided in two major groups, that is, the physical environment (temperature, oxygen, movements, etc.) and the medium composition.

Early attempts of culture have used high temperatures (39-40°C) with the rationale of mimicking the febrile conditions of clinical malaria [18, 34]. However, parasite growth at high temperatures was poor, a fact later confirmed when the optimal temperature for *P. vivax* development was established at 37°C [46].

Three different approaches have been used for the gas compositions: (i) candle jar, (ii) 5% CO_2, and (iii) the gas mixture of 90% N_2-5% CO_2-5% 0_2. Despite lack of evidence on its superiority, the 90% N_2-5% CO_2-5% 0_2 gas mixture is the most commonly used for both *P. falciparum* [56] and *P. vivax* cultures [29, 39, 47]. Oxygen concentrations below 5% or above 20% are fatal for the parasite [56], and a concentration of 5-10% has been shown to be optimal. Moreover, with 5% CO_2 at 37°C, cultures could be maintained for prolonged periods, though without effective parasite growth [38, 54].

Contradicting reports on the need of movement for culture (static versus non-static culture) have been published. For some authors movement is very important, particularly during the schizont stage to enhance merozoite release [40, 47], while other authors found movement deleterious, and parasites matured better in a static culture [36, 37]. Since good parasite growth has been observed in static cultures [40], movement is probably not a critical factor for *P. vivax* growth.

The medium composition is crucial for the culture as it should contain all necessary nutrients for the parasites to survive and multiply. Two basic media have been used for *P. vivax*: RPMI 1640, the most commonly used (reported in 11 out of the 15 publications on short and long-term cultures), and McCoy's medium (in 3 out of 15 publications). The composition of these two media is similar since McCoy's medium was derived from RPMI 1640. Indeed, it includes all additives as in RPMI 1640 (with slightly different concentrations) plus some additional vitamins and amino acids: (i) nicotinic acid (vitamin B3, involved in tissular respiration), (ii) ascorbic acid (vitamin C, anti-oxidant), (iii) pyridoxal (vitamin B6, involved in aerobic metabolism), and (iv) peptone (source of amino acids). Other media such as Locke's fluid [1], Waymouth 752/1, or SCMI 612 [19] have been tested and abandoned though Brockelman *et al.* [34] reported a better *P. vivax* multiplication with SCMI compared to RPMI medium. Similarly, the human serum concentration varied considerably between the 15 protocols (ranging from 10% to 50%) as did the haematocrit level (ranging from 3% to 25%), without any significant difference observed in terms of parasite growth and density.

Other *Plasmodium* species blood cycle *in vitro*

Among human *Plasmodium* species, the long-term *in vitro* culture of *P. falciparum* was published in 1975 by Trager and Jensen [14], and several laboratory adapted strains (3D7,

DD2, W2, among others) are currently available (www.mr4.org). Recently, a successful adaptation of *Plasmodium knowlesi*, a simian species infecting humans in Asia, to continuous culture in human erythrocytes has been reported by Moon *et al.* [57]. The culture was started with a frozen *P. knowlesi* A1 strain (previously maintained in rhesus macaques) supplemented with fresh RBCs from the natural host (*Maccacus fascicularis*) at 2% hematocrit in a modified RPMI medium containing 10% human serum, in static culture at 37°C. The strain was adapted over a 10-month period with a progressive change from monkey to human RBCs. Given the similarities between both *P. knowlesi* and *P. vivax* (for example, genetic, hosts, and target cells), this protocol could open new perspectives for the *in vitro* adaptation of *P. vivax*.

Other non-human *Plasmodium* species, (simian, rodent, avian) have been cultivated *in vitro* [58-63], and a long term culture system is available for four of them, that is *P. knowlesi*, *Plasmodium cynomolgi*, *Plasmodium gonderi* and *Plasmodium berghei* (Table 5). Useful clues for *P. vivax* culture may be obtained by sharing expertise gained from these non-human *Plasmodium* cultures, despite the fact that all of them use similar protocols as for the *P. falciparum* culture.

Table 5. Human and non-human *Plasmodium* species with published long-term *in vitro* cultures

Species	Host	Target blood cells	Medium	Other conditions: support[a], temperature, gas[b]	Blood source	Days in culture
P. falciparum	Human	RBCs	RPMI 1640 + HEPES + human serum	Flow apparatus, temperature: 37°C, gas: candle jar[c]	Human blood	Lab strains
P. knowlesi	Monkey, human	Reticulocytes	RPMI 1640 + HEPES + sodium bicarbonate, dextrose + hypoxanthine + AlbuMAX II, L-glutamine + gentamycin sulfate + human serum	24-well plate, temperature: 37°C, gas mixture 1[d]	Monkey and human blood	Several months
P. cynomolgi	Monkey	RBCs	RPMI 1640 + HEPES + human serum	16-mm flat-bottomed well, temperature: 37°C, gas: candle jar[c]	Monkey blood	34 days
P. gonderi	Monkey	RBCs	RPMI 1640 + HEPES, human/monkey serum	25 cm^2 flask, temperature: 37°C, gas: candle jar[c]	Monkey blood	6 months
P. berghei	Rodent	RBCs	RPMI 1640 + HEPES + heparin + $NaHCO_3$ + neomycin + FCS	Suspension culture apparatus, temperature: 37°C[e], gas mixture 2[f]	Mice blood	Well established

[a]Support: recipient in which the *in vitro* culture is done.
[b]Gas: gas environment provided to the parasite culture.
[c]Candle jar = 79% N_2 + 3% CO_2 + 18% O_2.
[d]Gas mixture 1 = 90% N_2 + 5% CO_2 + 5% O_2.
[e]The temperature was kept at 30°C for the first 20 h.
[f]Gas mixture 2 = 85% N_2 + 5% CO_2 + 10% O_2.

Concluding remarks:

Since the first attempt in 1912, the achievement of the reliable and continuous culture of *P. vivax* remains elusive, despite different culture conditions tested. The most puzzling aspect in this 100-year old history is the lack of reproducibility of the published protocols. Golenda and colleagues [40] were able to achieve a thriving culture, with parasite counts doubling at each schizogonic cycle, that was maintained for 15 days. Nevertheless, the reasons for discontinuing this apparently successful protocol after 15 days, as well as the inability of other research groups to reproduce it are unclear. This applies also to the protocol (125-day culture with a final parasite density of 8.6 %) published by Renapurkar *et al.* [45] in 1983. Potentially, the additional publication of negative results could be useful in understanding published positive results and in identifying unsuccessful protocol conditions. A large consortium including all research groups working on *P. vivax* culture in which all results, including the negative ones, would be shared and discussed could be extremely useful as it would avoid replicating non-viable protocols indefinitely.

In several reports, cultured parasites developed well only during the first schizogonic cycle, after which parasite density decreased dramatically. The reasons for such a drastic drop are unknown but may be related to the 'environmental shock' due to the sudden change from the host to *in vitro* conditions. The protocol by Golenda and colleagues [40] supports this hypothesis as the *P. vivax* Chesson strain was first maintained for several cycles in *Aotus* monkeys before adapting it to *in vitro* conditions by slowly and regularly supplying reticulocytes from hemochromatosis patients. Thus, a gentle adaptation, starting with *P. vivax* monkey-adapted strains and continuing with the gradual replacement of monkey blood with human reticulocytes could be, as recently shown for *P. knowlesi* [57], a possible solution.

A crucial step for the establishment of the long-term culture is the availability of a continuous and reliable source of target cells (reticulocytes). The *in vitro* invasion of reticulocytes by *P. vivax* may be affected by the concentration method. Indeed, centrifugation on Percoll renographin has been reported to interfere with the invasion [37] while this is not the case for Percoll alone [29], and ultracentrifugation in homologous plasma is likely to be even less disruptive for the invasion process [40]. Similarly, parasite invasion was less efficient with reticulocytes concentrated on Percoll than with those by MACS (Magnetic Activated Cell Sorting) [64]. It has been shown that i*n vitro* parasitized reticulocytes were very fragile, and

an early disruption of the membrane occurred before the end of the intra-erythrocytic maturation. Moreover, such *P. vivax* merozoites seemed to adhere to mature RBC membranes instead of infecting them, resulting in the rapid loss of parasites and reduced infection capacity [37].

To overcome these difficulties, the production of reticulocytes derived from HSC or ESC represents an interesting alternative since they would not be weakened by a Percoll concentration step. In addition, since the differentiation process produces a heterogeneous population of erythroid cells at different stages of differentiation, the maturation of orthochromatic erythroblasts into reticulocytes would occur at different time points, meaning a regular influx of new targets cells for *P. vivax* merozoites. This is a potentially interesting feature for maintaining a *P. vivax* culture even though orthochromatic erythroblasts themselves are known to be only poorly invaded by parasites [65].

ESCs have been previously differentiated into erythroid lineage using different protocols and are commercially available [66]. This is a major advantage as it would allow the development of a standardized, continuous and independent source of reticulocytes. However, the cost, and for some countries the need for special authorizations, could make this method less accessible. Mice stem cells and induced pluripotent stem cells (iPSC) could also be used, though their enucleation rate was reportedly lower than for ESC [67].

One potential problem of using HSC from cord blood or ESC/iPSC is that the produced reticulocytes contain foetal haemoglobin. Indeed, the presence of foetal haemoglobin has been reported to decrease the susceptibility of RBCs to *P. falciparum in vitro* [68]. However, this was contradicted by another report showing no difference in *P. vivax* maturation when using reticulocytes originating from cord blood or from hematochromatosis patients, the latter containing mainly adult haemoglobin [47]. The use of HSC from bone marrow (BM) or from peripheral blood may represent a good alternative as showed previously in a *P. falciparum* culture using BM HSC derived RBCs [69]. However, the question on the potential deleterious effect of foetal haemoglobin on the parasite growth still needs to be answered. This requires further studies comparing the intra-erythrocyte parasite development with foetal or adult haemoglobin.

Besides the availability and suitability of target cells, the use of an optimal culture medium with adequate supplementation is of major concern. Currently, the available knowledge on the biology of *P. vivax* blood stage parasites is inadequate to identify any missing or insufficient concentrations of nutrients. *P. vivax* culture conditions have often been based on the experience gained with *P. falciparum* cultures, though the two species differ in many aspects [70]. The difficulty in setting up an optimal culture medium for *P. vivax* is illustrated

by the often contradictory results obtained by different research groups and the obvious lack of continuity and reproducibility of published work. For instance, despite Brockelman's report on RPMI1640 being inferior to other culture media, this medium continued to be used [34]. The only conclusion on the most suitable culture medium is that the McCoy's medium supplemented with glucose is the minimum requirement for parasite growth. According to our own experience, other supplements (ascorbic acid, hypoxanthin, iron, and magnesium) do not seem essential for *P. vivax* growth, at least in the first *in vitro* maturation cycle (Celine Borlon, unpublished data). However, future studies are necessary to determine the optimal medium conditions for *P. vivax* growth, and in this respect, future metabolomic studies may help in identifying key elements in the parasite maturation.

In conclusion, after one century of efforts, research on a continuous *P. vivax* culture is as fragmentary and uncoordinated as ever. Rapid advances can be made only by sharing the accumulated knowledge, published and unpublished, for example negative results and comparing different protocols. A concerted effort is definitely needed for the ultimate goal of the long-term culture for *P. vivax.*

Chapter III: Stem cells

Stem cells are undifferentiated cells that can differentiate in different cell types as well as self-renew. We can distinguish 2 types of stem cells: embryonic stem cells (ESC) derived from the blastocyst and adult stem cells found in diverse adult tissues. Noteworthy, umbilical cord blood stem cells are also considered "adult" even if they are obviously obtained from newborns.
The characteristics of stem cells are that they can self-renew; the cells can multiply without losing their undifferentiated state. They are also characterized by different degrees of potency, i.e. which cell types they can develop into. Totipotent stem cells (the equivalent of the fertilized egg and the morula) can develop into all the embryonic and extra-embryonic cell types. Pluripotent stem cells (the equivalent of the inner cell mass of the embryo, and cell lines derived from this, such as ESCs) can develop into all the embryonic cell types (the three germ layers and gametes). Multipotent stem cells can be found in the different germ layers and in nearly all tissues. They are multipotent as they can generate all cells of a given tissue, but no longer cells from other tissues, such as the hematopoietic system, mesenchymal stem cells, neural stem cells, gastrointestinal stem cells, to name a few. Subsequently potency decreases further to oligopotent and unipotent stem/progenitor cells (such as the spermatogonial stem cell)

Human embryonic stem cells (hESC)

hESC are pluripotent cells that can, during embryogenesis, develop into 3 germ layers: endoderm, mesoderm and ectoderm. These 3 different germ layers will then be at the origin of different organs formation (fig.6). hESC are pluripotent cells derived from the inner cell mass of blastocyst-stage embryos fertilized *in vitro*. The pluripotency can be monitored by assessing the expression of specific transcription factors i.e. Oct-4, Sox-2, Nanog, by a compliment of specific cell surface antigens (in human TRA-160 and SSEA4). For murine ESC, proof that pluripotency requires that the cell can for chimeric animals, even when injected in tetraploid blastocysts. In human, this is obviously impossible to do, and the best test to demonstrate pluripotency is by teratoma assay where hESC are injected sub-cutaneous into immunodeficient mice. After 30 days, teratomas form and can then be analyzed using immunohistochemistry to demonstrate presence of the 3 germ layers.
Mouse ESC were first isolated in 1981 [71] and can be maintained in culture undifferentiated on a inactivated murine embryonic fibroblasts (iMEF) feeder layer with fetal bovine serum (FBS) and leukemia inhibitor factor (LIF)[72].

The first description of hESC was reported in 1998 [73] where Thomson et al, demonstrated that cells could be harvested from the ICM of human fibroblasts, expanded *in vitro*, while maintaining pluripotency characteristics, when cultured on iMEF with addition of specific growth factors [74]. The self-renewal of hESC is under the dependence of the transformer growth factor beta (TGFbeta)/activin/Nodal signaling [75] and basic fibroblast growth factor (bFGF) [76].

Notably, differentiated cells can be "undifferentiated" by forcing the expression of 4 pluripotent markers called Yamanaka factors: Oct-4, Sox-2, Klf-4 and c-Myc [77]. Those cells named induced pluripotent stem cells (iPSC) can be maintain and multiplied *in vitro* before been differentiated de novo.

One way of allowing differentiation of hESC is to allow them to form organoids, also termed embryoid bodies (EBs), where the ESC spontaneously differentiate in cells of the three germ layers. Much more difficult, however, is to create homogenous differentiated progeny in a coordinated manner. Even if we know many things about development and about the end cell one wishes to create from ESCs, most differentiation protocols to date, whether for neural, cardiac, hepatic or hematopoietic cells, fall short in creating transplantable cell populations, or even fully mature cells that would be suitable for disease in a dish studies, as in this thesis.

hESC could be used in many applications, especially for cell-based therapies. The production of hESC-derived organs or tissues could be of great interest in the frame of the transplantations that are still dependent of organ donors. Indeed, the generation of pancreatic cells producing insulin would be of great interest for peoples suffering of diabetes.

The development of iPSCs would be also of great interest in order to resolve the problem of histocompatibility by dedifferentiating cells from the patient in order to generate the necessary tissue or organ for an autologous transplantation.

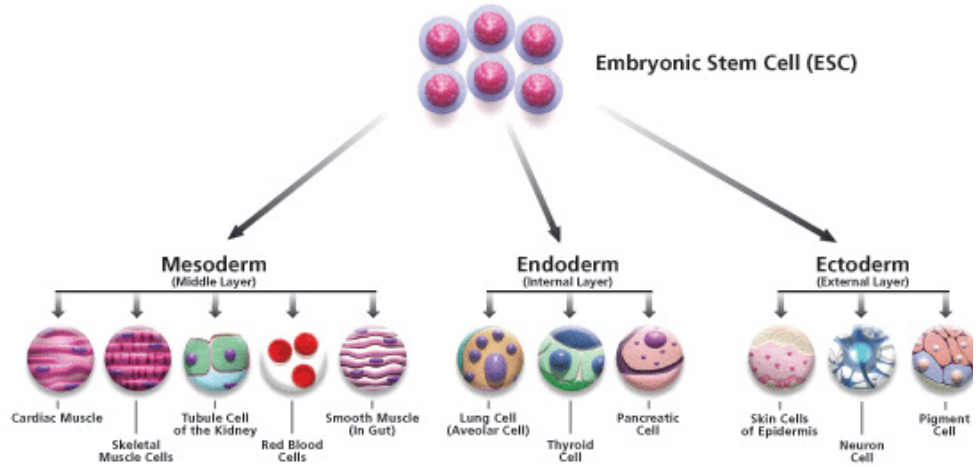

Fig. 6: Commitment of ESC into different germ layers and tissues.

Adult stem cells (somatic cells)

Adult stem cells are multipotent cells that can give rise to several types of cells but with a less broad range than ESCs. Their main function is the maintenance and reparation of the tissue where they are found, inside their stem cell niche [78]. The adult stem cells can stay quiescent (inactivated stage) until they will be activated due to a specific need of cell by the tissue for its reparation of for replacing cells after apoptosis [79].

Hematopoietic stem cells (HSC) are the most studied "adult" stem cells [80] and also commonly used for cell therapy against several blood diseases [81, 82]. Also many other somatic cells exist and are the origin of several cell types i.e. mesenchymal stem cells (osteoblasts, adipocytes, chondrocytes), neural stem cells (neurons, oligodendrocytes, astrocytes), dental pulp stem cells (teeth).

Somatic cells can also be used for therapeutic treatment i.e; mesenchymal stem cells for bone or cartilage repair or umbilical cord blood stem cells to treat blood disorders [83, 84].

Hematopoiesis

Hematopoiesis is the process wherein different mature blood cells are being produced from a multipotent stem cell named, hematopoietic stem cell (HSC). During the embryonic development, hemangioblasts that are precursors of HSC and endothelial cells will migrate from yolk sac to aorta-gonad-mesonephros (AGM) where the first HSC can be observed. HSC will then seed into fetal liver where they will mature and expand. During adult life, hematopoiesis will occur in the bone marrow (fig.7).

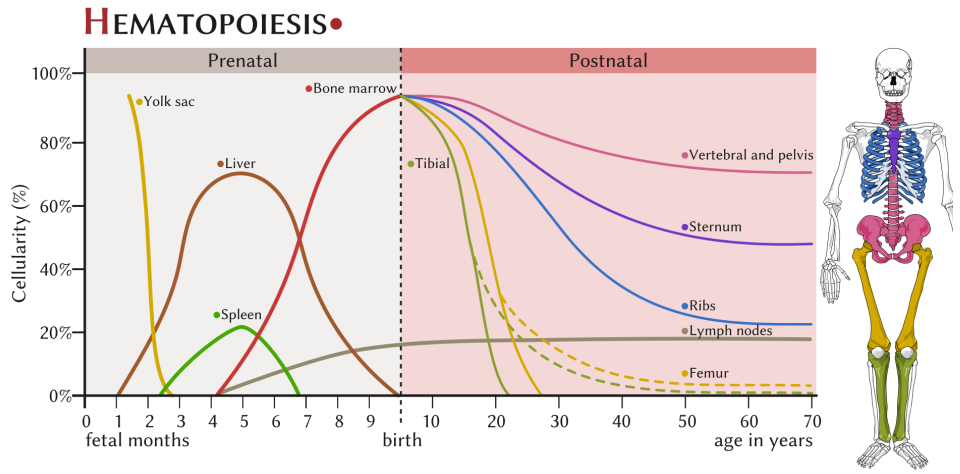

Fig 7: localization of the hematopoiesis process during life (figure from Michal Komorniczak)

Different stages of the erythroid development (fig.8)

If we focus on the erythroid development that concerns our work, the production of erythrocytes follows the following pattern starting from HSC that are present in the adult BM and very few in the PB. However at birth, when HSC migrate from the liver to BM, they can be found in large numbers in the umbilical cord blood (UCB). Their morphology is close to the lymphocyte one with an important nucleus and a very round shape.

We can distinguish 2 kinds of HSC: those that are able of self-renewal and restore hematopoietic system over some months called long term HSC and those that restore rapidly the hematopoietic system but are not able of self-renewal called short-term HSC. The specific surface markers to identify the HSC population are: $CD34^+$, $CD59^+$, CD90/Thy1$^+$, $CD38^{low/-}$, c-$Kit^{-/low}$, and Lin^-

Notably, the long term HSC have the capacity of self-renewal, which mean that they can multiply and keep their stemness. This process involves several genes that when expressed, direct the HSC to self-renew, i.e. Notch 1, Ikaros, HoxB4 or GATA-2 [85]. The expansion of HSC will also be under the influence of growth factors i.e. thrombopoietin (TPO), stem cell factor (SCF), interleukin 6 (IL-6) or FMS-like tyrosine kinase 3 ligand (FLT-3L) [86].

Following the HSC stage, we find the Colony Formation Units-Granulocyte, Erythrocyte, Monocyte, Megakaryocyte (CFU-GEMM) stage corresponding to the multipotential progenitor cells at the origin of the myeloid lineage. They can be characterized by the expression of $CD33^+$ and $CD34^+$

The CFU-GEMM will then evolve in burst forming unit-erythroid (BFU-E) cells that are the earliest erythroid precursor cells. They express on their surface the $CD33^+$, $CD34^+$ and HLA-DR. The colony-forming units erythroid (CFU-E) stage follows as they are more mature cells than BFU-E and their development is under the dependence of erythropoietin (EPO), and they express the $CD36^+$, $CA1^+$ and $CD71^+$ markers.

The earliest stage of the erythroid development is the pro-normoblast stage. These cells (12-20 µm) are characterized by an important nucleus, with multiple nucleoli, occupying 80% of the cell and a blue cytoplasm. They express on their surface the $CD36^+$, $CD71^+$, $CD117^+$, $CD235a^+$, and HLA-DR.

The following stage is the basophilic normoblast stage These cells (12-17 µm) are morphologically close to the pro-normoblasts with the exception that they do not have multiple nucleoli and they express the same surface markers excepted the $CD117^+$

The polychromatophilic normoblasts are the following stage; the nucleus becomes smaller compared with previous erythroid stage due to the chromatin condensation. The cytoplasm

starts to acquire hemoglobin, resulting in a more blue color. Their size is between 12 and 15 µm: and they do not express the HLA-DR unlike the basophilic normoblast

The orthochromatophilic normoblasts are the last stage of nucleated erythroid cells. The nucleus is condensed and the cytoplasm gets coloration close to the one of mature normoblast for a size of 8-12 µm and on expression on their surface of $CD36^+$, $CD71^+$ and $CD235a^+$ At this stage, cells will enucleate *in vivo* as a result of contact between the orthochromatophilic normoblast and a macrophage. Orthochromatophilic normoblasts interact with macrophages through an interaction between surface receptor (Vascular cell adhesion molecule 1: V-CAM 1, intercellular adhesion molecule: I-CAM 4 and erythroblast macrophage protein: EMP for macrophages and $\alpha_4\beta_1$, α_5 and EMP for orthochromatophilic normoblast) to form an erythroblastic island [87].

The first stage of erythroid cells without nucleus is named reticulocyte. The lifespan of reticulocytes is nearly 2 days (one day in the bone marrow and one day in the blood stream) and their express the CD235a+ and CD71+ markers, for a size of 7-9 µm.

Erythrocytes are the final erythroid stage. Erythrocytes are no longer round, but are bi-concave cells without nucleus or mitochondria for a size of 6-8 µm. Their lifespan is about 120 days and while it reaches this period, modification its membrane with the expression of phosphatidylserine on their surface, recognizable by macrophages that will phagocytize them in spleen, liver or lymphoid nodes. This phenomenon is called eryptosis. They express on their surface the $CD235a^+$ marker.

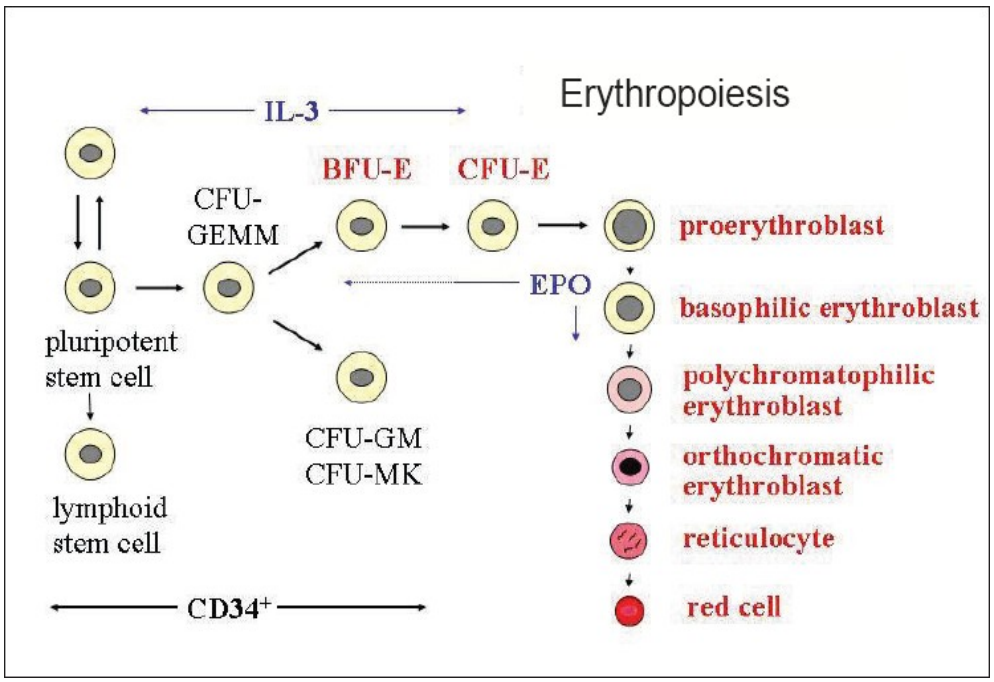

Fig.8: hematopoiesis and erythropoiesis lineage

Hemoglobin (Hb)

Hemoglobin is a quaternary structure with two kinds of polypeptide chains: alpha (α) and beta (β). It represents 97% of the dry erythrocyte content (35% of the total content) and contains a pocket that binds the heme group (fig.9).

Hemoglobin serves as an oxygen carrier from the respiratory organs to the rest of the body. Its capacity is 1.34 mL of O_2 per gram. Beside O_2, hemoglobin can carry in smaller quantity other gases: CO_2 and NO.

Fetal hemoglobin (HbF): mainly present during the last 7 months of fetus development and 6 months after birth. Whereas adult Hb (HbA) is composed of 2 α and 2 β chains, HbF is composed of 2 α and 2 γ chains.

HbF has a greater affinity for O_2 than HbA (fig.10) due to its lower interaction with 2,3-bisphosphoglycerate (2,3 DPB), molecule decreasing the affinity of Hb for O_2 [88].

During hematopoiesis, Hb is being expressed during the polychromatophilic normoblast stage and the switch between HbF and HbA occurred during the orthochromatophilic normoblast stage.

The switching mechanism from HbF to HbA is a unique phenomenon in animal reign as it can be found only in humans and old world monkey even if the mechanism underlying this switch is still poorly understood. A recent study has demonstrated that BCL11A is responsible for silencing γ globin expression [89], but how this is triggered is still unknown [90].

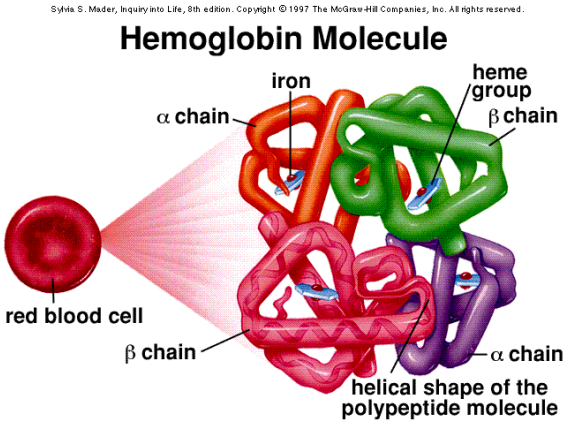

Fig.9: adult hemoglobin structure

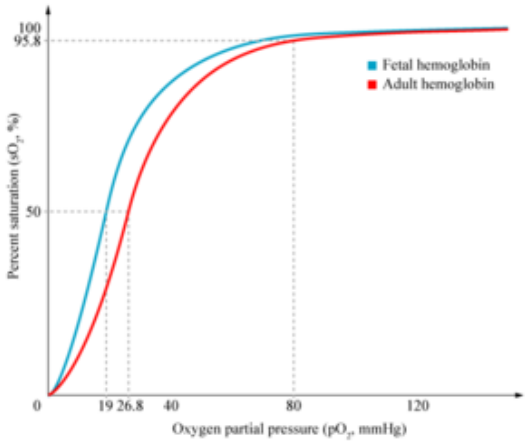

Fig.10: O_2 affinity curves for adult (red) and fetal (blue) hemoglobin. On the Y axis, the percentage of saturation and on the X axis, the O_2 pressure in mmHg

AIMS AND OBJECTIVES

The main goal of this work is to produce reticulocytes that are suitable for the invasion and maintenance of an *in vitro* culture of *P. vivax*.

- Objective 1: Produce reticulocytes derived from hematopoietic stem cells isolated from umbilical cord blood and test their permissiveness to *P. vivax*.

P. vivax needs reticulocytes as target cells, nevertheless we need to increase the quantity of reticulocytes to facilitate the meeting between the parasite and its host cell. Hematopoietic stem cells provide a good starting point to differentiate erythroid cells and produce reticulocytes in a sufficient amount to provide target cells the *in vitro* culture of *P. vivax*.

- Objective 2: Produce reticulocyte from different sources of hematopoietic stem cells and identify the best source for *P. vivax* culture.

Even if *P. vivax* can successfully invade UCB/$CD34^+$-derived reticulocytes, we cannot observe an *in vitro* multiplication of the parasite. We propose here to investigate different sources of HSC in order to identify which one could produce the most suitable cells to the parasite. Moreover we will try to increase the starting population of HSC from each source using a protocol of expansion to optimize the production of reticulocyte.

We will also investigate the influence of the hemoglobin stage as it has been previously described in the literature that fetal hemoglobin could slow down the intra-erythrocyte development of the parasite.

- Objective 3: To set up a reliable protocol of cryopreservation for the different reticulocytes produced.

P. vivax completes its blood life cycle in 48 hours and the reticulocyte maturate into mature erythrocyte in the same laps of time implying a frequent add of reticulocyte to support the multiplication of the parasite. Knowing that the full protocol of differentiation is 14 days, the possibility to create a stock of frozen reticulocytes could be a very interesting asset to handle the differentiation and the parasite culture in parallel.

- Objective 4: Produce reticulocytes from embryonic stem cells (ESC).

The possibility to use ESC as starting material for differentiation into reticulocytes could allow standardizing the protocol. Indeed, we would get an unlimited source of stem cells independent of inter-individual variations. Moreover, the possibility to genetically modify those cells could be of great interest to create reticulocytes more suitable for *P. vivax in vitro*.

CHAPTER I: Cryopreserved reticulocytes derived from hematopoietic stem cells can be invaded by cryopreserved *Plasmodium vivax* isolates

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Cryopreserved Reticulocytes Derived from Hematopoietic Stem Cells Can Be Invaded by Cryopreserved *Plasmodium vivax* Isolates

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Abstract

The development of a system for the continuous culture of *Plasmodium vivax in vitro* would benefit from the use of reticulocytes derived from differentiated hematopoietic stem cells (HCS). At present, the need to use both fresh reticulocytes and fresh *P. vivax* isolates represents a major obstacle towards this goal, particularly for laboratories located in non-endemic countries. Here, we describe a new method for the cryopreservation of HSC-derived reticulocytes to be used for both *P. falciparum* and *P. vivax* invasion tests. Cryopreserved *P. falciparum* and *P. vivax* isolates could invade both fresh and cryopreserved HSC-derived reticulocytes with similar efficiency. This new technique allows the storage of HSC-derived reticulocytes which can be used for later invasion tests and represents an important step towards the establishment of a continuous *P. vivax* culture.

Keywords: Hematopoietic stem cells, reticulocytes, *Plasmodium vivax*, Cryopreservation, invasion tests

Introduction

Outside sub-Saharan Africa, *Plasmodium vivax* is the most common human plasmodium species [1]. Though it has long been considered as less dangerous than *P. falciparum*, severe and fatal *P. vivax* cases as well as drug resistant parasites are now increasingly reported [2,3,4,5]. Understanding of the biology and the transmission dynamics of *P. vivax* lags behind that of *P. falciparum*, partly because the maintenance of a continuous line of this parasite *in vitro* has not been established. The availability of an *in vitro* culture system would improved our understanding of the biology of *P. vivax*, e.g. invasion and relapse mechanisms, resistance markers, and would contribute to the development of new treatments and vaccines.

P. vivax preferentially invades reticulocytes [6] and thus in order to achieve a continuous *in vitro* culture system the availability of large amounts of these young red blood cells, which circulate in the peripheral blood at low concentration (1% of total red blood cells) and for a very short time (24h), is required. Russell *et al.* [7] recently published a reliable invasion assay protocol using fresh *P. vivax* schizonts from fresh clinical isolates and enriched cord blood reticulocytes. It has been previously shown that reticulocytes can also be successfully produced through the differentiation of hematopoietic stem cells (HSC) [8] and that such HSC-derived reticulocytes may be used for *P. vivax* culture, though both the reticulocyte production and the parasite densities obtained were extremely low [9]. The contribution of this paper is to report an improved method to produce and cryopreserve HSC-derived reticulocytes to be later invaded by *P. vivax*, allowing research on *P. vivax* culture to be carried out outside endemic areas, increasing the number of teams potentially working on this subject and hence the chances for major discoveries.

Materials & Methods:

Ethics statement:

P. vivax sample collection: MUTM 2008-15 from the ethics Committee of the faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand

Cord blood sample collection: blood was collected anonymously and patients were informed orally with a possibility of opting-out. Each Patient was notified on the hospital admission form of this opting-out possibility.

Procedure was accepted by ethic committee of UZA and ITM.

Study was approved by the ITM review board, number: SBB.219.2007/1410

Hematopoietic stem cell (HSC) isolation. Umbilical cord blood samples (40ml each) were collected from pregnant women delivering at the University hospital, Antwerp (UZA), after obtaining an individual informed consent. Mononuclear cells (MNC) were separated by Ficoll-Isopaque (GE Healthcare) centrifugation (250 g, 10 min) and enriched for $CD34^+$ cells by supermagnetic microbead selection using Mini-MACS columns (Miltenyi Biotech) according to a previously published procedure [10].

HSC culture. The amplification procedure was adapted from a three-step expansion of $CD34^+$ cells by sequential supply of the culture with specific combination of cytokines and growth factors [11]. HSCs isolated from cord blood were cultured at 37°C, 5% CO_2 in a modified serum-free media (IMDM, Biochrom) supplemented with L- Glutamine (4 mM, Sigma), Penicilline/ Streptomycine (1 %, Invitrogen), Inositol (40 µg/ml, Sigma), Folic acid (10µg/ml, Sigma), Monothioglycerol ($1.6\ 10^{-4}$ M, Sigma), Transferrin (120 µg/ml, Sigma), insulin (10 µg/ml, Sigma), Bovine Serum Albumin detoxified by beads resin AG501-X8 (Biorad) (BSA, 100 mg/ml, PAA) [12].

Step 1 (day 0 to 8): $CD34^+$ cells were cultured with Stem Cell Factor (SCF, 100 ng/ml, Bioke), IL-3 (5 ng/ml, R&D System), Hydrocortisone (HDS, 10^{-6} M, Sigma), and

Erythropoietin (EPO, 3 IU/ml, R&D System). At day 4, cells were diluted 1:2 in IMDM medium completed with all the four above mentioned growth factors and incubated at 37°C for 4 additional days. At day 7, cells were re-suspended (10^6 cells per vial) in culture medium (IMDM) and an equal volume of 80% Foetal Calf Serum (FCS)/20% DMSO solution was added drop by drop to obtain an IMDM/40% FCS /10% DMSO solution before progressively freezing them at -80°C using a Mr Frosty [13].

Step 2 (day 8-11): at day 8, 250 000 cells were added to each $25cm^2$ flask and incubated in 5 ml of IMDM medium supplemented with only EPO (3 IU/mL).

Step 3 (day 11-20): the culture was maintained in IMDM without growth factors or cytokines and the medium was changed every 3 days. The culture was stopped at day 14 corresponding to the peak of reticulocytes counts determined by microscopic examination of thin films done by cytospin (Thermo scientific): 200 000 cells were washed with PBS once and re-suspended in 50 µL of PBS. 50 µL of Cresyl Blue(Merck)(previously diluted 1/100) were added to the tube and incubated 30 minutes. FCS (30 µL) was added to protect cells during cytospin centrifugation and the cells were placed in a cytospin funnel. After a centrifugation (700 rpm, 3 minutes), slides were removed from the funnel and stained with Giemsa. A reticulocyte scoring positive would contain two or more blue-stained RNA granules.

HSC cryopreservation. HSC derived reticulocytes at day 14 in the culture were subjected to three different cryopreservation protocols:

Glycerolyte solution [14]: 100 µL of Glycerolyte (Baxter) were drawn up to the cell pellet using an insulin syringe. Firstly, 20% of the volume of Glycerolyte was added to the blood cell suspension drop by drop while continuously agitating the tube to mix the content. This suspension was incubated 5 minutes at room temperature before adding the rest of Glycerolyte. RBC-Glycerolyte mixture was then aliquoted into cryovials and frozen at -80 °C overnight before being stored in liquid nitrogen.

Glycerol & Sorbitol solution [15]: Cells were frozen in a medium composed of 28% glycerol, 3% sorbitol, and 0,9% NaCl diluted in distillate water. This solution was added drop by drop to packed cells, at room temperature. Cells were immediately transferred at -80°C.

IMDM/10% DMSO/40% FCS solution[16]: Cells were frozen according to a standard method with medium containing 10% DMSO/40% FCS as described above for the HSC culture at day 7.

Reticulocytes were kept in liquid nitrogen up to 1 year. Cells were thawed following the same protocol as for *Plasmodium falciparum*: the volume (V) of the thawed content was measured and transferred to a 50ml centrifuge tube. Then 0.1 V of pre-warmed (37°C) 12 % NaCl solution was added drop by drop to the parasitized red blood cells (pRBC), which were allowed to stay for 5 min at room temperature. Subsequently 10 V of 1.6 % NaCl solution (pre-warmed at 37 °C) were carefully added to the cells, which were then centrifuged for 10 min at 650 g without applying a break. The supernatant was discarded and 10 ml of 0.9% NaCl solution pre-warmed at 37 °C was carefully added to the pellet, followed by a 5 min centrifugation at 650 g [17]. The cryopreservation protocols were evaluated according to the following criteria: viability of the cells after thawing, preservation of the reticulocyte population, presence or absence of clots, and presence of invaded reticulocytes by *Plasmodium*.

FACS analysis. HSCs cultures were analysed by flow cytometer at day 8, 11, 14 and 17 of their in-vitro maturation. After thawing the cryopreserved reticulocytes they were analysed by FACS, to assess the expression of stage specific markers: CD36, CD45, CD71 and CD235a. Briefly, 200 000 cells were centrifuged for 5 min at 650 g. The pellet was re-suspended in 100µL of PBS buffer. 5 µl of CD45-PE (BD bioscience), 10 µL of CD71-APC (BD bioscience), 5 µL of CD36-FITC (BD bioscience), and 5 µL of CD235-PerCP (BD bioscience), were added to the cells and incubated for 15 min at room temperature in the dark. One tube containing only antibody isotypes coupled to fluorochromes was used as a negative control: 10 µL of IgG1γ-PE (BD bioscience), 5 µL of IgM-FITC (BD

bioscience), 5 µL of IgG1 K isotype-PerCP (BD bioscience), and 5 µL of IgG1 K isotype-APC (BD bioscience). After incubation cells were washed with FACS buffer (Miltenyi Biotech) and re-suspended in 500 µL of buffer before analysis. FACS analysis were carried out on a FACScalibur 4 color cytometer (BD bioscience).

Hemoglobin content. The haemoglobin (Hb) content (foetal or adult haemoglobin) of the HSC derived reticulocytes was analyzed using the Kleihauer method [18]. Umbilical cord blood (≥95% of foetal Hb) and adult peripheral blood (≥95% of adult Hb) were used as controls. Cells containing foetal haemoglobin will be stained in red whereas cells containing adult haemoglobin will look empty and only their membranes will be observed.

Plasmodium falciparum. *P. falciparum* D10 strains, cultured and cryopreserved in the Institute of Tropical Medicine, Antwerp, Belgium, (ITMA) cryobank were used for this study. Parasite isolates were thawed in a 37°C incubator until no ice was left in the tube. The thawing protocol was the same as the one used for reticulocytes. To check for the invasion of new RBCs (RBC derived from HSC maturation) by P. falciparum, pRBC were concentrated on a Percoll gradient (90 %, 70 %, and 40 %; Sigma) [19] and centrifuged for 30 min at 2600g without applying a break. After centrifugation, the layer between the 70 % and 40 % Percoll was kept, transferred into a new tube and washed with RPMI (Lonza). 1 µl of purified pRBC (≥ 90% mature forms) was mixed with 4 µL of the HSCs derived reticulocytes and 94 µL of RPMI medium supplemented with HEPES (25 mM, Lonza), L-glutamine (1 mM), gentamycin (40 µg/ml, Sigma), D-glucose (2 %, Sigma), hypoxanthine (50 mg/L, Sigma) diluted in NaOH 1M and 10 % heat inactivated Human Serum (final volume: 100 µL, haematocrit 5 %). The culture was incubated in a hermetic chamber flushed gas (90% N2, 5% CO2, 5% O_2) at 37°C for 24 h. Parasite invasion was determined by microscopic examination of Giemsa stained thin films. The number of newly invaded cells by *P. falciparum* ring stages were counted and divided by the total number of cells to obtain the parasite density (% of invaded RBC). For each experiment, two technicians independently examined the slides and the mean parasite density was

taken as the final value . If reading results were discordant (parasite counting difference ≥30%), both technicians re-read the slide until an agreement was found.

Plasmodium vivax. *P. vivax* isolates were collected from patients with acute P. vivax malaria (mono-infection with a density of >1parasite/1000 red blood cells) attending the clinics of the Shoklo Malaria Research Unit (SMRU, Mae Sot, North-West Thailand), after obtaining their individual written informed consent. P. vivax isolates contained more than 80% early ring forms. White blood cells were removed after filtration on CF11 column (Whatmann) [20]; Parasites were frozen in Glycerolyte solution [21], as described above, and then transferred and kept in liquid nitrogen at the ITMA. Subsequently, *P. vivax* isolates were thawed following the same protocol as described above for P. falciparum, and were re-suspended in pre-warmed 37°C McCoy's 5A (Gibco) medium supplemented with 20% heat inactivated human serum from normal AB group donors and 0.5% D-glucose. The medium containing *P. vivax* was then transferred to a 25 cm^2 culture flask, flushed with gas (90% N2, 5% CO2, 5% O_2), and placed in a 37°C incubator for 36 to 44 hours to allow the maturation into schizonts. Parasite stage was determined by microscopic examination of Giemsa stained thick and thin films. Mature schizonts were concentrated in 45% Percoll by centrifugation for 15 minutes at 1580 g without applying a break (Sigma)[22]. Purified schizonts (≥ 90% mature forms) were mixed with HSCs derived reticulocytes resulting in a starting parasite density between 0.5 and 1 %. Complete Mc Coy's medium was added in order to obtain a 5% haematocrit level and the culture was incubated at 37°C in a hermetic chamber flushed for 24h with gas (90% N_2, 5% CO_2, 5% O_2) [7]. The efficiency of invasion was checked as described above for *P. falciparum.*

Data analysis: During HSC maturation, reticulocytes and RBC were counted for each selected day during the maturation process. Median value and interquartile range (IQR) were calculated for the reticulocytes counts (6 independent experiments) as well as for the expression of the surface markers measured by FACS (3 independent experiments).

Parasite densities for *P. vivax* invasion assays were calculated per 500 counted cells. The number of infected cells was divided by the total number of counted cells and multiplied by 100 to obtain a percentage.

Results:

Amplification of erythroid cells. Microscopic examination of thin films of the erythroid cells (Figure 1) confirmed a normal differentiation pattern and the commitment of the HSC culture to the erythroid lineage as defined elsewhere [23]. A total of 30 independent experiments were successfully carried out, and median reticulocyte and RBC population counts were computed at regular intervals (every 3 days between day 8 and day 20) for 6 experiments (Figure 2). The first reticulocytes were observed at day 12 of differentiation, and their number dramatically increased between days 13 and 14 (from 5% to 18%), corresponding to a peak of enucleation. Subsequently, the percentage of reticulocytes decreased steadily (10% at day 16) and almost disappeared at day 19 of differentiation. The haemoglobin content measured at day 14 of maturation was mainly foetal (about 90 %, data not shown). Mature red blood cells (RBC) showed an opposite trend as compared to reticulocytes as they started to increase following the reticulocyte peak (0.4% at day 15), outnumbered the reticulocytes at day 17 and reached a plateau of above 20% at day 20. After cryopreservation, the cells viability was up to 70 % and the proportion of reticulocytes remained stable compared to the one before freezing (except for glycerolyte which was not tested due to the presence of clots during the thawing process).

FACS analysis of HSC maturation. The proportion of positive cells expressing different surface markers was calculated at different time points (Figure 3a). The CD45, a specific marker for young cells (pre-proerythroblast stage), decreased dramatically from 90% at day 8 to 3% at day 11, while CD235a (Glycophorin A marker) followed the opposite trend. Both CD71 and CD36 (both markers of stages between the proerytroblast and mature RBC) were highly expressed at day 8, 100% and 80% respectively, and decreased following a different pattern until day 17: the CD71 decreased slightly to 65% at day 17, while the CD36 dramatically decreased to less than 10% at day 17. A freezing cycle (up to 1 year in the frame of this work) of the HSC derived reticulocytes which were matured till day 14 did not affect the expression of specific reticulocytes

markers for any of the three protocols tested. Indeed the comparison with the freshly produced HSC derived reticulocytes gave similar results: (Figure 3b) a high expression of CD71 (between 95 % and 98 % according to the freezing protocol *versus* 90% for fresh reticulocytes at day 14), and CD235a (between 90 and 98 % *versus* 90 % in fresh reticulocytes), a quasi-absence of CD45 (around 0.5 % *versus* 1%), and the expression of CD36 reaching 30 % for the 3 protocols tested (*versus* 40%).

Invasion assays. Both *P. falciparum* and *P. vivax* could successfully invade freshly HSC derived reticulocytes (Figure 4a and 4b). Parasite density after invasion of fresh reticulocytes was around 0.5 % for *P. vivax* and 4% for *P. falciparum*. Parasite invasion was then tested on the cryopreserved reticulocytes derived from HSC maturation. *P. falciparum* could invade cryopreserved reticulocytes subjected to any of the 3 cryopreservation methods (parasite density: 4.2 % for glycerol solution, 4.1% for Glycerolyte and 8% for the IMDM/10% DMSO/40% FCS; 3 independent assays). However, the method using the Glycerolyte solution was discarded as clots were observed during the thawing process. Therefore, for *P. vivax* invasion, only reticulocytes cryopreserved with the Glycerol solution and the Medium/10% DMSO/40% FCS solutions were used. These were successfully invaded with the identification of ring stages 24h post invasion and the parasite density was counted for all 8 independent experiments (Figure 5). *P. falciparum* and *P. vivax* could invade the erythroblasts stages, with both fresh and cryopreserved HSC derived cells (Figure 4c).

Parasite density obtained after invasion of cryopreserved HSC derived reticulocytes varied substantially (from 0.19% to 2.43%) according to the *P. vivax* isolate used. However, parasite density for each single parasite isolate did not vary when using different batches of cryopreserved reticulocytes. The range of parasite densities after invasion was between 0.19 % and 2.43 % for reticulocyte cryopreserved in the IMDM/10% DMSO/ 40% FCS solution. In comparison, invasion rate of reticulocytes cryopreserved in glycerol solution was 30 to 50 % less efficient in 4 replicates) (Figure 5).

Discussion

This is the first report showing a reliable cryopreservation protocol for HSC-derived reticulocytes derived that can be successfully invaded by both cryopreserved *P. falciparum* and *P. vivax* isolates. This will contribute to the establishment of a continuous *P. vivax* culture, since the latter requires a continuous supply of reticulocytes in a large amount. Until now, availability of adequate quantities of reticulocytes was a bottleneck, as they represent only 1% of all circulating cells in the blood stream [24]. Several approaches have been tested to tackle this problem. One used blood from hematochromatosis patients [25] as they need to be regularly bled and have a higher percentage of reticulocytes. This method allowed for short-term *P. vivax in vitro* cultures for up to 15 days. However, the limited access to hemochromatosis patients is a drawback. A second source of reticulocytes is cord blood, in which the reticulocyte concentration is slightly higher (3-8 %) than in adult blood; this source has been previously used permitted maintaining *P. vivax* fresh isolates in culture for about one month [26]. However, the low amount of reticulocytes obtained did not allow for an optimal parasite multiplication (parasite density <0.7 %). Moreover, substantial variation of parasite densities obtained makes this protocol unsuitable for studying *P. vivax* biology.

Cord blood HSCs represent a valuable source, as reticulocytes can be produced in substantial quantities [9,27]. The differentiation protocol presented here was adapted from the one published by Giarratana *et al.* [8,28], which originally matured HSCs into erythroid lineage by using a co-culture with stromal cells. The latter are mainly used to retain nucleated cells, free nuclei and debris so that the culture appears cleaner. We have modified this technique by omitting the co-culture with stromal cells, which resulted in a less pure population of enucleated cells in our model (20% enucleated/80% nucleated cells *versus* 100 % enucleated cells when using stromal cells) since the nucleated cells were not retained by the MS5 layer. Nevertheless, while

Giarratana aimed at obtaining RBCs with a lifespan of about 120 days, achieved during the later stages of RBC maturation, our purpose was to obtain reticulocytes whose lifespan is 2 days. This explains why maturation was stopped at the peak of the reticulocyte concentration (D14). Moreover, reticulocytes obtained after maturation on a MS5 layer gave similar result in term of invasion than those produced without stromal cells (data not shown). A previous attempt of producing HSC-derived reticulocytes without using stromal cells in co-culture attained a maximum reticulocyte concentration of 0.5% [9]. This is in contrast with the results of our methods which produced a 40-fold (20%) higher concentration of reticulocytes. The low efficiency of the previous method could be explained by several factors such as the use of non-detoxified BSA (a key factor for the last steps of maturation and enucleation) [27], a very high (lethal?) concentration of monothioglycerol, or the absence of inositol or folic acid. It is also important to mention that the parasite density in culture supported by HSC-derived reticulocytes obtained with our method was substantially higher (2.5% vs 0.0015 %) than that reported for the previous one [9], providing sufficient biological material to possibly keep the culture for a longer period.

During the invasion assays we performed, the cell population was not purely composed by reticulocytes but contained also erythroblasts. Both *P. falciparum* and *P. vivax* were able to invade these cells, as previously reported [29]. No information on the quality of the parasite development in this type of cells is currently available.

FACS analyses of both fresh and cryopreserved reticulocytes showed the expression of CD235a marker (Glycophorin A), an important receptor for *P. falciparum* invasion [30]. Its role in *P. vivax* invasion has not been described yet but its homology with the Duffy Binding Protein (DBP) [31,32] suggests a potential role in the *P. vivax* invasion process of the reticulocytes. Thus the cryopreservation protocol kept the important receptors on the reticulocyte membrane.

The added value of the work presented is the set up of a cryopreservation technique for the long-term storage of cord blood HSC-derived reticulocytes, providing a continuous source for the performance of invasion assays and possibly the maintenance of a *P. vivax*

in vitro culture. The technique is robust as it has been tested on several HSCs batches of that gave comparable results with a single *P. vivax* isolates (Fig.5, PV3/ CB 81 and 23). The reticulocyte cryopreservation using Glycerolyte was abandoned due to the presence of clots during thawing. It is unclear why this occurred but a possible hypothesis would be the harmful effect of glycerolyte on nucleated cells.

The source of reticulocytes for a *P. vivax* invasion assay is not limited to HSC-derived cells. Borlon *et al* [32] were able to maintain a short-term (up to 10 days) *P. vivax* culture using reticulocytes directly concentrated from cord blood. This was done with similar efficacy with both fresh and frozen parasites and reticulocytes (using Glycerolyte 57 as cryopreservant). The advantage of concentrating reticulocytes from cord blood is that these are almost immediately available (pending availability of cord blood) while HSC-derived ones need 14 days to mature. Nevertheless, with the former technique the concentration process (percoll centrifugation, application of several washing/concentration steps) could damage the cells and affect the invasion efficiency while using HSC-derived reticulocytes may guarantee a more homogeneous and standardised cell population. Eventually, using both methods: concentrated from cord blood and HSC-derived reticulocytes could substantially increase the amount of available reticulocytes from a single cord blood sample.

In conclusion, the cryopreservation method described in this paper provides a continuous and substantial source of reticulocytes, possibly a first step to achieve the continuous *in vitro* culture of *P. vivax*. In addition, the possibility of cryopreserving both reticulocytes and parasites offers the opportunity of investigating P. vivax invasion assays/ culture in non-endemic countries, opening this field of research to more research groups thus increasing the probability of fast advances in knowledge.

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Legend figures:

Figure 1: Production of reticulocytes from HSCs, morphologic analysis after Giemsa staining. (A) Human CD34+ cells isolated from cord blood (B) Day 4 of culture, (C) Day 8- (D) Day 11- (E) Day 14 of culture, reticulocytes are highlighted by cresyl blue staining (arrows).

Figure 2: Evolution of reticulocytes (continuous line) and red blood cells (dotted line) counts from day 12 to day 20 of HSCs maturation. Each value represents the median a total of 6 independent experiments, and vertical bars represent the interquartile range (IQR). Reticulocytes were identified by cresyl blue staining.

Figure 3: Expression of reticulocyte surface markers by flow cytometry.**3a**: Expression of HSCs surface markers at different maturation stages characterized by flow cytometry. Cells were collected, respectively at 8 days, 11 days, 14 days and 17 days of maturation and stained with specific antibodies against CD36, CD45, CD71 and CD325a receptors, respectively. Each value represents the median percentage for 3 independent experiments (vertical bars represent the extreme values in this case). **3b**: Comparison of the surface markers expression (%) at day 14 after thawing for 3 different cryopreservation protocols compared to fresh HSC derived reticulocytes (median % values of 3 independent experiments; vertical bars represent the extreme values in this case).

Figure 4: Pictures of HSC-derived reticulocyte invaded by *Plasmodium*.**4a**: Infected enucleated cells by *P. falciparum* 24h after invasion. (A) Fresh reticulocytes derived from HSCs maturation, (B) Cryopreserved reticulocytes with glycerolyte, (C) Cryopreserved reticulocytes with glycerol solution, (D) Cryopreserved reticulocytes with Medium /40% FCS/10% DMSO (3 replicates). **4b**: Infected reticulocytes by *P. vivax* 24h after invasion. (A) Fresh reticulocytes derived from HSCs maturation, (B) Cryopreserved reticulocytes with glycerol solution, (C) Cryopreserved reticulocytes with Medium /40% FCS / 10% DMSO (8 replicates)**. 4c**: Infected erythroblast (A) By *P. falciparum*. (B) By *P. vivax* (8 replicates).

Figure 5: Parasitemia of infected HSC derived cells for the 2 protocols of cryopreservation (Medium/10%DMSO/40%FCS and Glycerol solution) 24h after invasion assay with *P. vivax* cryopreserved isolates.

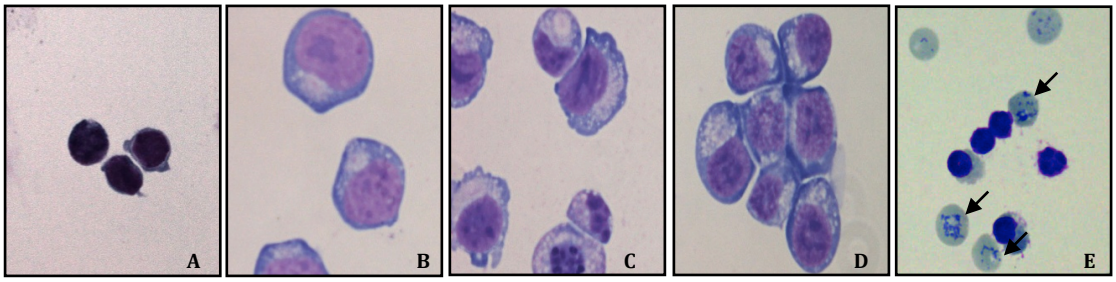

Fig 1. Production of reticulocytes from HSCs, morphologic analysis after Giemsa staining. (A) Human CD34+ cells isolated from cord blood (B) Day 4 of culture, (C) Day 8- (D) Day 11- (E) Day 14 of culture, reticulocytes are highlighted by Cresyl blue staining (arrows).

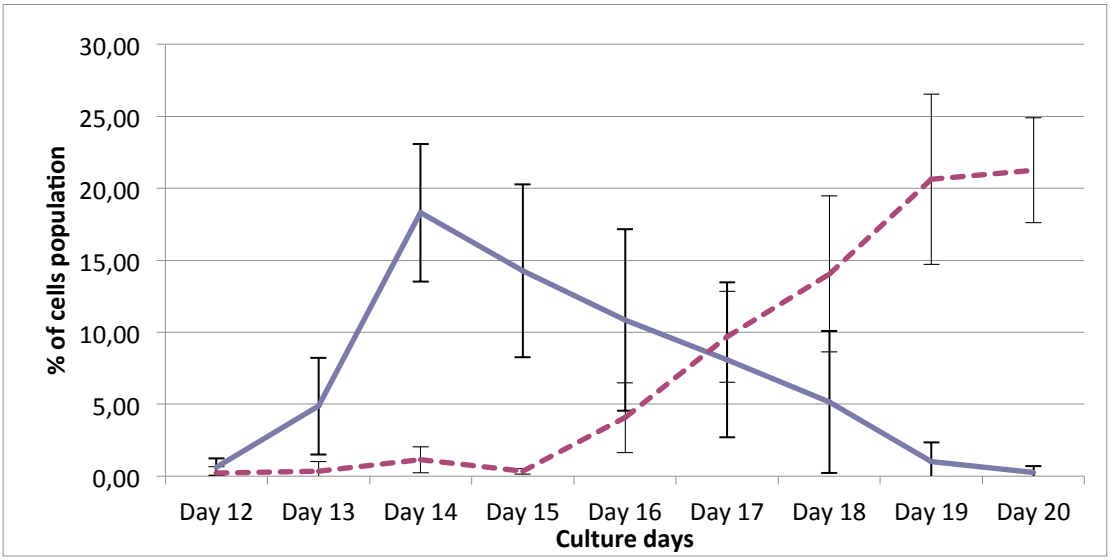

Fig 2. Reticulocytes were identified by Cresyl blue staining. Reticulocytes (continuous line) and red blood cells (dotted line) counts (vertical bars represent standard deviation) from day 12 to day 20 of HSCs maturation (total 6 independent experiments)

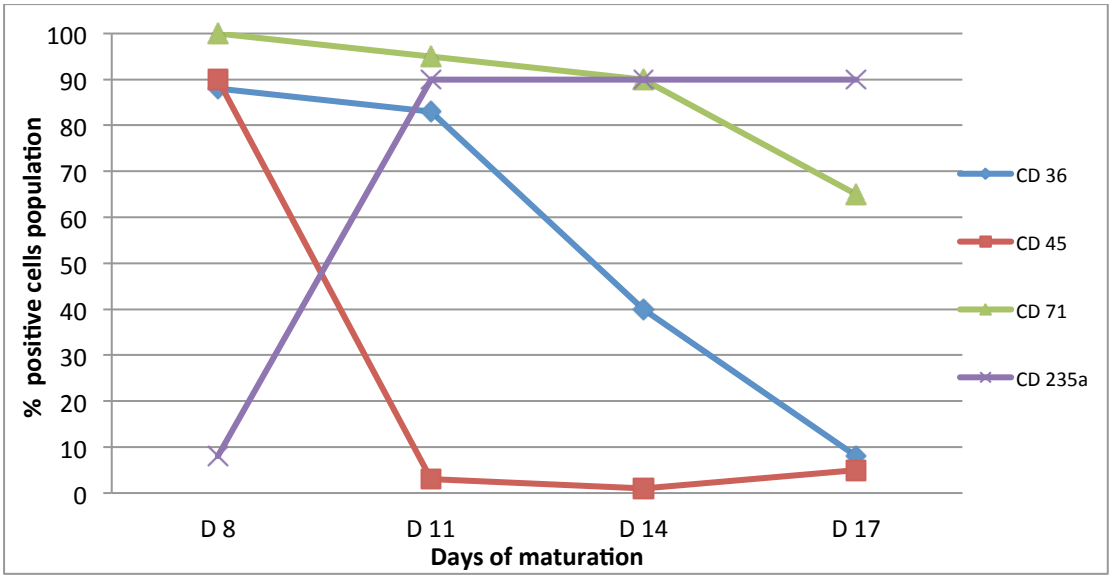

Fig 3a. Expression of HSCs surface markers at different maturation stages characterized by flow cytometry. Cells were collected, respectively at 8 days, 11 days, 14 days and 17 days of maturation and stained with specific antibodies against CD36, CD45, CD71 and CD325a receptors, respectively. Each value represents the median percentage for 3 independent experiments.

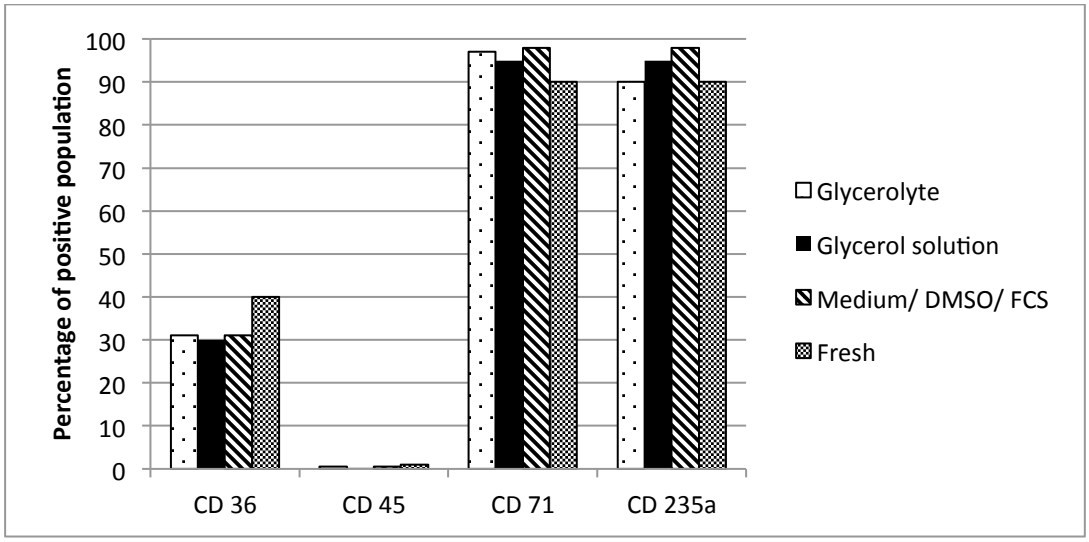

Fig 3b. Comparison of the surface markers expression (%) at day 14 after thawing for 3 different cryopreservation protocols compared to fresh HSC derived reticulocytes (3 independent experiments).

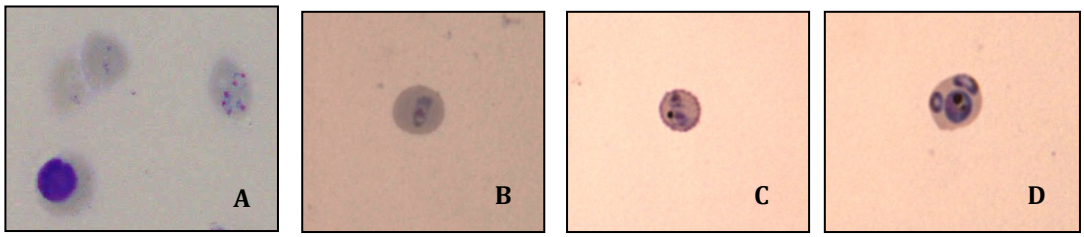

Fig 4a. Infected enucleated cells by *P. falciparum* 24h after invasion.
(A) Fresh reticulocytes derived from HSCs maturation, (B) Cryopreserved reticulocytes with glycerolyte, (C) Cryopreserved reticulocytes with glycerol solution, (D) Cryopreserved reticulocytes with Medium /40% FCS / 10% DMSO (3 replicates)

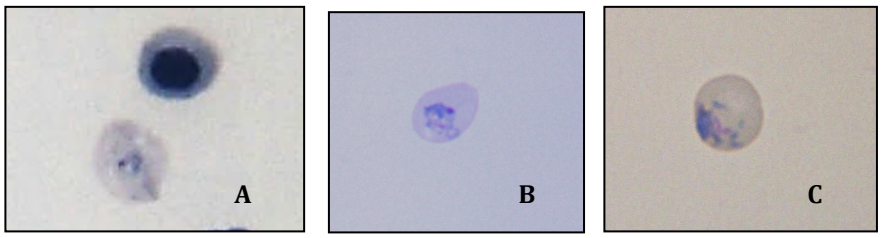

Fig 4b. Infected reticulocytes by *P. vivax* 24h after invasion.
(A) Fresh reticulocytes derived from HSCs maturation, (B) Cryopreserved reticulocytes with glycerol solution, (C) Cryopreserved reticulocytes with Medium /40% FCS / 10% DMSO (8 replicates)

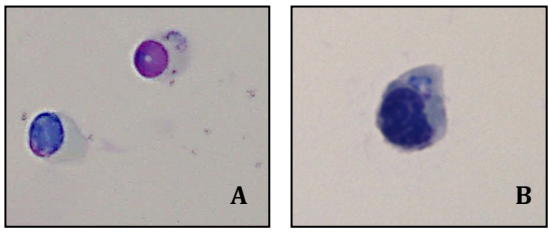

Fig 4c. Infected erythroblast (A) By *P. falciparum*. (B) By *P. vivax* (8 replicates)

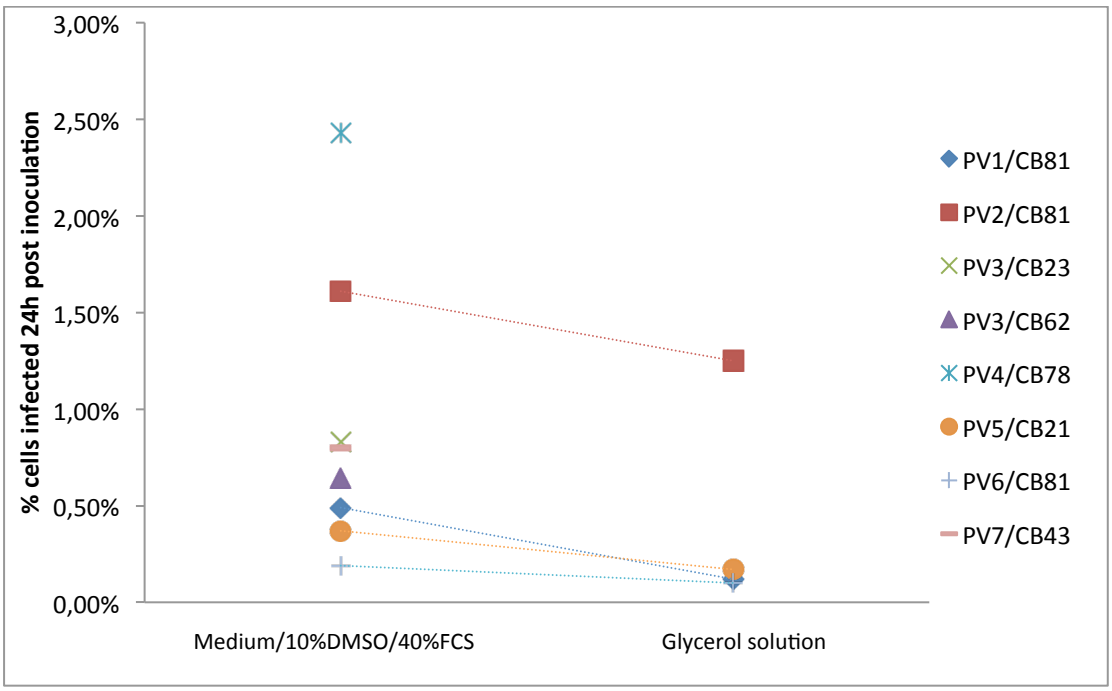

Fig 5. Parasitemia of infected HSC derived cells for the 2 protocols of cryopreservation (Medium/10%DMSO/40%FCS and Glycerol solution) 24h after invasion assay with *P. vivax* cryopreserved isolates.

CHAPTER II: Influence of $CD34^+$ source and hemoglobin type for culturing *Plasmodium* parasites

This chapter is currently under review in the Journal of Parasitology

Influence of CD34+ source and hemoglobin type for culturing *Plasmodium* parasites

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Running title: CD34+ cells and hemoglobin for *Plasmodium* culture

SUMMARY

The affinity of *Plasmodium vivax* (*P. vivax*) for reticulocytes constitutes a major hurdle for its *in vitro* culture. Hematopoietic stem progenitor cells (HSPC) have been used to produce reticulocytes; however, this has not yet enabled the establishment of a long term *P. vivax* culture. The main objective of our study was to assess if the presence of fetal hemoglobin (HbF) impedes the *in vitro* development of *Plasmodium falciparum* (*P. falciparum*), as well as the reticulocytes invasion by *P. vivax*. The second objective was to determine which source of HSPC would generate the largest yield of reticulocytes. Our results showed that HbF did not affect the erythrocyte invasion by *P. vivax* or *P. falciparum*, neither did it influence the *in vitro* development of *P. falciparum.* . Moreover, the *ex-vivo* expansion of umbilical cord blood HSPCs yielded in significantly higher amounts of reticulocytes compared to either peripheral blood or bone marrow HSPCs.

INTRODUCTION

Plasmodium vivax (*P. vivax*) is the most widespread malaria parasite outside Africa, and accounts for 80 to 300 million of malaria cases per year (Price et al., 2007). In 2010, 50% of all malaria cases outside Africa were due to *P. vivax* and occurred mainly in South-East Asia and South America (WHO report 2012). Control measures in areas where both *P. falciparum* and *P. vivax* occur have had a major impact on the burden of *P. falciparum* infections, but less so on *P. vivax*, indicating its "resistance" to currently available control methods developed mainly against *P. falciparum* (Feachem et al., 2010). This is due to a number of factors among which the existence of *P. vivax* dormant liver stages (hypnozoites) causing relapsing infections months or years after the primary infection, and the early production of *P. vivax* gametocytes before the infection becomes symptomatic. Moreover, the specificity of *P. vivax* for preferentially invading reticulocytes constitutes one of the bottlenecks for its establishment in long-term cultures (Moreno-Perez et al., 2013; Noulin et al., 2013). Indeed, reticulocytes represent 1% of the circulating red blood cells (RBCs) and have a half-life of 2 days (including 1 day in the peripheral blood), therefore they are difficult to obtain in sufficient amount to sustain the *in vitro* development of *P. vivax* .

Two approaches have been used to increase the number of reticulocytes both with limited success. One is the concentration of reticulocytes from adult or cord blood samples using either a 70% percoll solution (Russell et al., 2011)or a plasma autologous ultra-centrifugation (Golenda et al., 1997). The second approach is based on *in vitro*

culture of CD34+ cells to induce erythroid differentiation (supplementary figure 1), and hence produce reticulocytes. One study has reported a *P. vivax* culture for up to 85 days in reticulocytes differentiated from umbilical cord blood (UCB)-hematopoietic stem/progenitor cells (HSPC), but parasite densities were very low (Panichakul et al., 2007). We recently showed that *P. vivax* parasites appeared not to develop beyond one schizogony cycle when cultured in UCB/CD34+ derived reticulocytes (Noulin et al., 2012). Recently, reticulocytes derived from CD34+ cells isolated from bone marrow (BM) and peripheral blood mononuclear cells (PBMC) were shown to be permissive to *P. vivax* but no details were given about the efficiency of invasion and the quality of the parasites' *in vitro* development (Fernandez-Becerra et al., 2013a).

Many authors have argued that *P. vivax* failure to growth *in vitro* when using cord blood as source of reticulocytes (either by concentration or derived from HSPC), could be attributed, at least partially, to the presence of fetal hemoglobin (HbF) in the cord blood reticulocytes (Borlon et al., 2012; Noulin et al., 2012; Panichakul et al., 2007). This hypothesis is based on earlier work published, by Pasvol *et al* in 1977 (Pasvol et al., 1977) in which the authors compared *P. falciparum* invasion and parasite development in adult blood and blood from patients with hereditary persistence of fetal hemoglobin (HPFH). They observed that the invasion rate was the same for both RBC sources; however *P. falciparum* growth was delayed in the presence of HbF. Therefore, the authors concluded that the presence HbF negatively affected the development of *P. falciparum*, possibly due to the fact that *P. falciparum* parasites use hemoglobin also as a source of amino acids during their development (Sherman, 1977), besides the fixation

of 0_2 molecules. The question on the influence of HbF on *P. falciparum* in vitro development has not been revisited since then, and the same relationship has been assumed for *P. vivax*, possibly explaining its poor *in vitro* multiplication obtained so far.

Apart from UCB, other sources of multipotent $CD34^+$ cells, such as BM or peripheral blood (PB) can be used to generate reticulocytes. These "adult" sources of HSPC, unlike the UCB HSPC, differentiate into reticulocytes and RBCs that do not contain HbF (Fernandez-Becerra et al., 2013b).

The objectives of the current study were to assess whether the presence of HbF influenced the reticulocyte invasion by *P. vivax* or by *P. falciparum* and, for the later species, the *in vitro* development. The second objective was to determine which source of HPSC, ie UCB, BM, or PB, would be best to produce highest yields of reticulocytes.

RESULTS

Reticulocyte production and characterization

We obtained reticulocytes differentiated from magnetically sorted CD34-enriched populations originating from three different sources, i.e. UCB, PBMC and BM. We observed a peak of enucleation after 14 days of differentiation for all three sources of cells. Enucleation of erythroid cells from PBMC (mean=31%, SD±6) was significantly higher (p=0.002) than that of UCB (18%, SD±1.4) and BM (21%, SD±1.5) (Table 1).

BM and PBMC CD34+ enriched cells produced reticulocytes contained HbA while those derived from UCB $CD34^+$ cell-derived reticulocytes contained HbF, as assessed using the Kleihauer method. The concentration of hemoglobin per cell (or Mean Corpuscular Hemoglobin; MCH) was higher in all three groups of *in vitro* generated reticulocytes, i.e. 50, 60 and 39 pg/cell respectively for UCB, BM and PBMC, compared to the normal *in vivo* range i.e. 29.5-33.5 pg/cell.

HSPC expansion

The enrichment for $CD34^+$ cells in the sorted populations was 55% (SD±6), 35% (SD±8) and 16% (SD±6) for UCB, BM and PBMC respectively (n=3). After 5 days of expansion, the total cell populations increased by more than 10-fold in cultures seeded with UCB CD34+ cells, 3-fold for BM cells and no expansion was observed for PBMC (Table 2). FACS analyses showed an increase in the $CD34^+/CD45^+$ population for all three sources: from 55 % at D0 to 70% (SD±2) at D5 for UCB, 35% to 55% (SD±5) for BM, and 16% to 29% (SD±16) for PBMC (n=3 for CB and BM, n=2 for PBMC)(Figure 1)

Following expansion, a similar number of cells (irrespective of the CD34+ content or expansion) were dispensed in a 6-wells plate to induce reticulocyte differentiation. Following erythrocyte differentiation step, the total cell number was 3 times higher for $CD34^+$ cells from either source, compared with $CD34^+$ cells that were immediately induced into differentiation. After 12 days of differentiation, expanded cells expressed more than 80% of glycophorin A in all three groups of HPSC sources (data not shown). We observed a 5 to 10-fold higher proportion of reticulocytes at day 14 for the cells derived from *in vitro* expanded $CD34^+$ cells *vs.* cells differentiated directly from unexpanded cells for the different sources of HSPC.

P. falciparum invasion and intra-erythrocyte development

In order to determine which factors affect invasion and development of *P. falciparum* in erythrocytes, we used different sources of erythrocytes: i) derived from $CD34^+$ cells from CB, BM and PBMC, ii) directly from normal adult donors (HbA) peripheral blood,iii) directlyfrom one adult donor with β thalassemia minor (HbA, but lower Hb concentration per cell), iv) and from UCB (HbF). We followed the intra-erythrocyte development of *P. falciparum* every day for 7 days. *P. falciparum* invaded and developed in erythrocytes obtained from the three HSPC sources as well as in peripheral blood from normal adult donor, β thalassemia minor and from UCB for 7 days (figure 2). *P. falciparum* cultures became asynchronous, after 5 days when cultured with PB from normal adult donors (62% of rings and 28% of schizonts), and after 7 days when cultured with the β thalassemia minor patient's blood (63% rings and 27% schizont),

while the 48-hour cycle was still maintained up to day 7 when cultured with UCB (84% of rings stages and 14% schizont stages). Moreover, the multiplication ratio (number of new rings stage from each schizont stage burst) was comparable for the 3 sources of blood i.e. UCB, PB, and β thalassemia minor patient (around 3-fold at each schizogony cycle). The same experiments were repeated with 20% O_2, which resulted in a substantial reduction of parasite growth in PB from normal adult after 3 days compared to cultures in 5% 0_2 (3.9% versus 1.1%) and a growth deficiency of in presence of HbF.

P. falciparum could also invade and develop in HSPC-derived erythrocytes (expanded or not) for 7 days (synchronous culture up to day 7; data not shown). However, the invasion rate was lower than with UCB or adult blood as the proportion of target cells (enucleated cells+ orthoerythroblasts) was 30-40 % while in whole blood (UCB, PB or β thalassemia minor) all cells (reticulocytes+ erythrocytes) can potentially be invaded.

P. vivax invasion and intra-erythrocyte development

We also tested if reticulocytes derived from $CD34^+$ cells from the UCB, BM or PBMC could be invaded by *P. vivax.* As shown in figure 3, reticulocytes generated *in vitro* from $CD34^+$ cells or directly enriched blood (PB or UCB) could both be invaded. When using the same *P. vivax* isolate, the invasion rate did not differ between different HSPC sources (figure 4a). Nevertheless, when using the same HSPC-derived reticulocytes with two different *P. vivax* isolates, the invasion rate varied by isolate (figure 4b; PV1 and PV2, $p<0.001$). After 3 days, only few rings (parasite density < 0.05%) could be observed and none survived longer than 72 hours, regardless of the HSPC source. Interestingly, HSC-

derived reticulocytes seemed more vulnerable to *P. vivax* invasion than reticulocytes from enriched blood. Indeed, when using the same *P. vivax* isolate and comparing to HSPC-derived reticulocytes, the parasite density 24 hours post-invasion was up to 9-fold lower for UCB-concentrated reticulocytes (PV2: 1.8% *versus* 0.2%, respectively), and 18-fold lower for adult peripheral blood-concentrated reticulocytes (PV2: 2.1% *versus* 0.1% respectively) (supplementary data 2). The parasite densities were not significantly different between HSPC-derived reticulocytes (5%) and UCB concentrated reticulocytes (4.6% p=0.056) and PB concentrated reticulocytes (3.5% p=0.06) for a reticulocyte proportion of 20% for HSPC-derived reticulocyte versus 70% and 60% for UCB and PB concentrated reticulocytes respectively..

DISCUSSION

The present study investigated the influence of HbF on the *in vitro* development of *plasmodium* and tried to identify the best source of HSPC to produce reticulocytes. We showed that when magnetically sorted $CD34^+$ cells from either PBMC, BM or UCB are used to produce reticulocytes for *in vitro* culture of *P. vivax*, no significant difference is observed in the efficiency whereby the parasite invades the reticulocytes. However, none of the currently available systems supported the full development and long-term culture of *P. vivax*. Moreover, we showed that the invasion efficiency depended on the *P. vivax* isolate. Our results also suggest that the number of reticulocytes derived from UCB $CD34^+$ enriched cell populations can be substantially increased when the $CD34^+$ cells are first expanded for 5 days yielding a 30-fold higher reticulocyte number following differentiation.

The ability to substantially increase the number of reticulocytes generated per $CD34^+$ cell from UCB, BM and PBMC (30-fold UCB, 9-fold BM), using a simple 5-day protocol, is of great interest for the development of *P. vivax in vitro* culture. We previously demonstrated that reticulocytes can be cryopreserved (Noulin et al., 2012) and then used for *P. vivax* invasion. Noteworthy, when magnetically sorted PBMC/ $CD34^+$ cells that were cultured in similar expansion culture conditions showed a limited increase of HSPC compared to CB/HSPC and BM/HSPC. This is likely due to the fact that the frequency of $CD34^+$ cell in non-mobilized PBMC is low (Bender et al., 1994) and that

PBMC may be less immature than cells from UCB and BM respectively (Steidl et al., 2002).

The experiments clearly showed that the type of Hb in reticulocytes/erythrocytes did not affect the invasion of *P. vivax* or *P. falciparum,* neither did it influence the *in vitro* development of *P. falciparum*. . Unlike Pasvol *et al* (Pasvol et al., 1977), who reported that HbF negatively affects the development of *P. falciparum* in culture. Our experiments showed that the presence of HbF or lower levels of HbA do not affect *P. falciparum* invasion and development. Moreover, we did observe that the time to asynchronous culture of the *P. falciparum* culture was 5 days when reticulocytes/erythrocytes from PBMC were used, while this occurred only after 7 days for the beta thalassemia blood (lower HbA concentration) and after 7 days, the culture was still synchronous in the presence of UCB (HbF).

These results may be explained by the different gas composition used during parasite culture: while Pasvol *et al* using a gas composition of 75% N_2, 5% CO_2 and 20% O_2, the gas composition in our cultures (and commonly used for *P. falciparum* culture (Butcher, 1979)) was 90% N_2, 5% $C0_2$ and 5% O_2. This relatively lower O_2 concentration may have decreased the influence of the type of hemoglobin in the target cells as confirmed by the poor multiplication of *P. falciparum* in presence of 20% O_2.

The presence of HbF did also not affect the ability of the *P. vivax* to invade freshly isolated reticulocytes or reticulocytes derived from UCB $CD34^+$ cells. However,

irrespective of the origin of the cell source (PBMC, BM or UCB) and hence the type of Hb present, few *P. vivax* parasites could mature *in vitro* and invade new reticulocytes. This is not surprising, since long term *in vitro* culture of *P. vivax* has never been fully achieved so far despite numerous reports of successful invasions never followed by maturation to subsequent schizogony cycles with parasite expansion (Noulin et al., 2013).

Our preliminary observations could indicate a marked preference of *P. vivax* for immature reticulocytes as we could observe a more important permissiveness of our reticulocytes derived from any HSPC source compared to those concentrated from either UCB or PB. The more important invasion rate in reticulocytes concentrated from CB compared to those from PB could be explained by the distribution of the reticulocyte populations they contain. Indeed, Paterakis *et al* (Paterakis et al., 1993) classified reticulocytes according to their RNA content by FACS analysis and divided them into 3 categories: immature reticulocytes (high amount of RNA), median and old reticulocytes (medum and low amount of RNA respectively). They found that in the global reticulocyte population, UCB contains more immature reticulocytes (13,6%) than adult peripheral blood (1%). Furthermore, the HSPC-derived reticulocyte, they were collected at the peak of enucleation meaning that they were very immature reticulocytes. This could reinforce the idea of a preference of *P. vivax* for immature reticulocytes. Interestingly, when reticulocyte-enriched blood from UCB and PB were used at 3-fold higher reticulocyte concentrations (70 % and 60%, respectively), the invasion rates became similar to those obtained with HSPC-derived reticulocytes (20 % for HSPC). This is in line with a recently published report by Martín-Jaular *et al* (Martin-Jaular et al.,

2013) in which the authors could observed a predominant invasion of $CD71^{high}$-expression cells (CD71 being a marker of reticulocyte maturation) by *P. yoelii* (a mouse parasite that also invade preferentially reticulocytes). All these observations need further in-depth investigations that could provide new insights into the invasion mechanisms of *P. vivax* and more specifically on the critical stage-specific receptors. If confirmed, this hypothesis would justify the use of reticulocytes derived from HSPC instead of reticulocyte-enriched blood as target cells for the establishment of continuous *P. vivax* culture.

In conclusion, the presented results are of particular interest as they shed a new light on the possibility of producing large amounts of reticulocytes, which can be efficiently invaded by *P. vivax*. The ability to derive reticulocytes from HSPCs offers the advantage of developing a standardized and continuous source of reticulocytes required for the long term *P. vivax* culture.

Experimental procedures

CD34+ cell isolation: The differentiation of HSPC to reticulocytes has been previously described (Giarratana et al., 2005). Umbilical cord blood (UCB) samples were obtained from the Belgian Cord Blood Bank at the Gasthuisberg Hospital Leuven. Bone marrow (BM) samples were obtained from volunteer donors and human peripheral blood (PB) was obtained from the Antwerp Red Cross. Mononuclear cells from PB (PBMC), UCB and BM were isolated on a Ficoll gradient (GE Healthcare) by centrifugation for 30 minutes at 400 g. The mononuclear cells were collected and washed twice with PBS. Hematopoietic stem/progenitor cells (HSPC) were isolated by $CD34^+$ Magnetic Assorting Cell Sorting (MACS, Biotenyl biotech) as per manufacturer recommendations. Cell purity was assessed by FACS, using CD34 and CD45 antibodies (ebioscence).

Differentiation towards reticulocytes: CD34 enriched cells were dispended in a 6 wells plates with IMDM medium (Gibco) supplemented with L-glutamine (4M, Sigma), penicillin-streptomycin (1%, Invitrogen), folic acid (10 µg/mL, Sigma), inositol (40 µg/mL, Sigma), transferrin (120 µg/mL, Sigma), monothioglycerol ($1.6\ 10^{-4}$ M, Sigma), insulin (10 mg/mL, Sigma) and 10% human plasma. During the first 8 days, the medium was supplemented with specific growth factors: hydrocortisone (HDS, 10^{-6} M, Sigma), interleukin-3 (IL-3, 5 ng/mL, R&D system), stem cell factor (SCF, 100 ng/mL, Bioke) and erythropoietin (EPO, 3 IU/mL, R&D system) and placed at 37°C in a 5% CO_2 incubator. The initial volume of medium was 4 mL and after 4 days, an extra 3 mL was added. After 8 days, the cells were centrifuged for 5 minutes at 300 g, fresh IMDM medium

supplemented with EPO (3 IU/mL) was added and the cells were transferred in a flask 25 cm^2. At day 11, the medium was changed and only complete IMDM medium was added. Afterwards, medium was changed every 3 days and 10% heat inactivated human serum was added to protect cells against destruction.

*CD34+ cell expansion (*supplementary data *3)*: $CD34^+$ enriched cells were dispensed in a 6 well plate with 4 mL SFEM medium (Sigma) with SCF (50 ng/mL), TPO (50 ng/mL, R&D system), FLT3 (50 ng/mL, R&D system) and IL-6 (50 ng/ mL, R&D system) for 5 days at 37°C, 5% CO_2. At day 5, the cells were counted and transferred into a new 6 well plate to induce reticulocyte differentiation.

Reticulocyte counting: Cells were centrifuged at 300 g for 5 minutes and re-suspended in 50 µL of PBS, 50 µL of Cresyl blue (Roche) diluted 1:1000 was added and cells were incubated at room temperature for 30 minutes. After cytospin centrifugation (3 minutes at 700 rpm), cells were fixed with methanol, and stained for 10 minutes with Giemsa (Sigma). Slides were then examined by microscopy (immersion objective, 630X magnification) and the reticulocytes were counted (a reticulocyte was defined as an enucleated cell with at least 3 spots of cresyl blue RNA was defined as a reticulocyte).

Hemoglobin (Hb) stages were defined using the Kleihauer method (Kleihauer and Betke, 1960), based on elution and specific Hb staining. Briefly, a thin smear was made and fixed 10 minutes with 80% ethanol. After drying, the slide was incubated 5 minutes with elution buffer pre-warmed at 37°C. The next steps were a primary staining of 3 minutes with Mayer's hemalyum and after washing a 2^{nd} staining with 0.1% eosin. The slides

were then examined by microscopy and cells enumerated: the cells containing HbF were red stained while those containing HbA were not stained ("ghost cells") enumerated.

The Hb concentration of Hb per cell was measured using the hemocue method. The mean corpuscular Hb (MCH) was calculated by dividing the measured Hb concentration measured by the number of enucleated cells.

Plasmodium invasion assays

- *P. falciparum*: A vial of the laboratory strain 3D7 (cryobank, ITM Antwerp) was thawed as per the well-established protocol (Blomqvist, 2008). The parasites were maintained in culture with RPMI medium (Lonza) supplemented with Hepes (25 mM, Lonza), L-glutamine (1 mM), gentamycin 40 µg/mL, Sigma), glucose (2%, Sigma), NaOH (1M), hypoxanthine (5O mg/mL, Sigma), and 10% heat inactivated human serum in a 37°C incubator with a gas mixture composed of 90% N_2, 5%0_2, 5% CO_2. Mature forms of *P. falciparum* (schizonts) were concentrated on a percoll gradient (4mL 90% percoll, 2 mL 70% percoll, 2 mL 40% percoll). After centrifugation, the layer between the 70% and 40% percoll, was transferred in a new tube and washed twice. A thin smear was done to check the efficiency of the concentration and when the population of parasitized RBCs (pRBCs) was over 90%, cells were mixed with the target cells in a 96 wells plate and the initial parasitemia adjusted to 0.5-1% (final volume 100 µL, hematocrit 2-5%). The plate was placed in a 37°C incubator with 90% N_2, 5% 0_2, 5%

CO_2. The parasitemia was checked daily on a microscopy slide made using cytospin (3 minutes at 700 rpm) and giemsa staining.

- *P. vivax isolates and invasion assays* (supplementary data 4): Cryopreserved *P. vivax* isolates (Rossan, 1985) from patient were provided by the Shoklo malaria research unit (SMRU, Mae Sot, Thailand). The samples were thawed with a NaCl solutions and cultivated for 30 hours with McCoy's medium (Gibco) supplemented with glucose (2%) and 20% human serum heat inactivated. Mature forms were concentrated on a 45% percoll after a 5 minutes treatment with 0.05% trypsin. After 15 minutes of centrifugation at 1600g, cells above the 45% percoll were collected and washed twice before checking the quality of the concentration. If more than 90 % of the cells contained parasites, they were mixed with our target cells (containing the same percentage of reticulocyte for all) in a 96 wells plate and adjusted the initial parasitemia on a 1:6 ratio (final volume 100 µL, hematocrit 2-5%). Cells were checked at 24 hours post-invasion by cytospin slide and giemsa staining.

Data analyses:

Data were entered and cleaned in Excel (version?); data analyzed was performed with STATA12. Reticulocytes were counted after 14 days of differentiation and the mean ±SD calculated for each source of HSPC. The Kruskall-wallis test was used to test for significant differences between the three population means. Parasite densities were counted for 500 cells and the number of infected cells divided by the total number of

cells to obtain of percentage of infection. The mean and standard deviation were calculated for HSPC expansion rates.

Ethics statement:

P. vivax collection: samples collection was approved by the ethics committees of the faculty of tropical medicine, Mahidol university, Bangkok, Thailand (number MUTM-2008-15) and the University of Oxford, Centre for Clinical Vaccinology and Tropical Medicine, United Kingdom (number: OXTREC 027-025) .

Cord blood samples: samples were collected from the cord blood bank at the Gasthuisberg hospital, Leuven, Belgium: ethical approval number ML6620

Bone marrow sample were taken from voluntary donors at the Gasthuisberg hospital, Leuven, Belgium: ethical approval number B322201112107

Adult peripheral blood: blood samples were bought from the Antwerp Red Cross blood bank

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Authors declare having no conflict of interest

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Figure legends:

Table 1: Comparison of the differentiation output for the different hematopoietic stem progenitor cells (HSPC) sources (6 independent experiments for each HSPC source). Results show the median value and interquartile range (IQR) of the proportion of reticulocytes observed at the peak of enucleation after 14 days of culture. The hemoglobin stage was checked by Kleihauer method and the concentration of hemoglobin per cells was measured by the Hemocue method.

Table 2: Comparison of the cell expansion rate at different time points for the different hematopoietic stem progenitor cells (HSPC) sources. The number of cells was counted after 5 days of expansion and divided by the initial number of plated MACS/CD34+ cells to assess expansion rate. After 7 days of HSPC differentiation, the number of cells was counted and compared to the cells that were not expanded in step 1. The enucleation rate was also calculated by divided the number of reticulocytes obtained after 14 days of differentiation for cells that underwent the expansion step and cells that were differentiated without prior expansion. The average of 3 independent experiments and the standard deviation were calculated and reported in this table.

Figure 1: FACS analyses of the CD34+ cells from UCB, PBMNC and BM, after isolation and following 5 days of expansion. The Q2 gate represents the population double positive for

CD34 and CD45. We observed an increase $CD34^{+}APC/CD45^{+}PE$ frequency in all three cases.

Figure 2: Proportion of the different stages of *P. falciparum* in culture on a daily base. The proportion of rings (blue), trophozoites (red), schizonts (green) and gametocytes (purple) was counted and adjusted to 100 parasites to obtain the proportion of each stage. The average of 3 independent experiments has been calculated for each day of the culture. The *P. falciparum* culture become asynchronous after 5 days in presence of adult peripheral blood, after7 days in presence of beta thalassemia blood, while with cord blood it remained synchronous up to day 7.

Figure 3: Cytospin of the HSPC-derived reticulocytes with by P. vivax 24h post invasion. Cells were stained with Giemsa. a) UCB/HSPC-derived reticulocytes b) BM/HSPC-derived reticulocytes; c) PBMC/HSPC-derived reticulocytes.

Figure 4: Parasite densities 24 hours post-invasion with *P. vivax*. a) comparison of parasites density of *P. vivax* 24 hours post-invasion for the different HSPC sources and reticulocytes concentrated from blood for a same parasite isolate. b) parasite densities for 4 different *P. vivax* isolates with different reticulocyte sources. PV1 and PV2 were tested with the same HSPC-derived reticulocyte. The parasite density was counted for at least 500 cells, dividing the number of infected cells by the total number of cells.

Table 1: Reticulocyte differentiation in 3 different sources of HSPC

HSPC source	Mean reticulocyte proportion at D14°	Hb stage	Hb concentration (pg/cell)°*
UCB	18.3% [17-19]	Fetal	60[50-70]
BM	20.5% [20-21]	Adult	60.5 [60-61]
PBMC	32% [29-32]	Adult	44.5 [39-50]

° Proportion of reticulocytes (%) with interquartile range [IQR]
*Range Hb concentration in vivo: (29.5-33.5 pg/cell)

Table 2: Cell expansion rate at different time points for the three sources of HSPC. Results are expressed in mean± SD fold increase

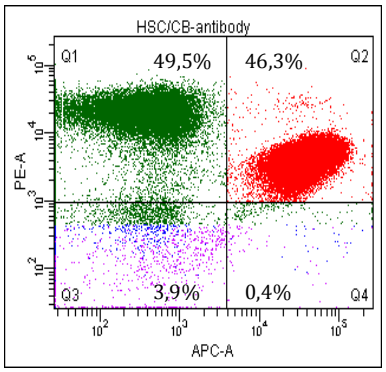
	Following 5 days of expansion	Following 7 days of differentiation
UCB	11.5± 2.3	33.5 + 2.4
BM	3.1 ± 0.3	8.6 ± 0.5
PBMC	1.3 ± 0.2	3.4 ± 0.2

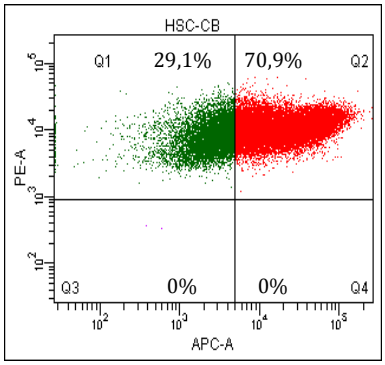
Figure 1: FACS analyses of the CD34+ cells from UCB, PBMNC and BM, after isolation (day 0) and after 5 days of expansion.

D0 of expansion

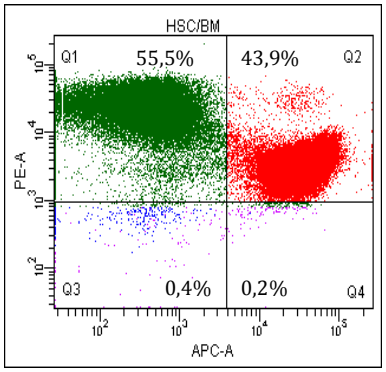
D5 of expansion

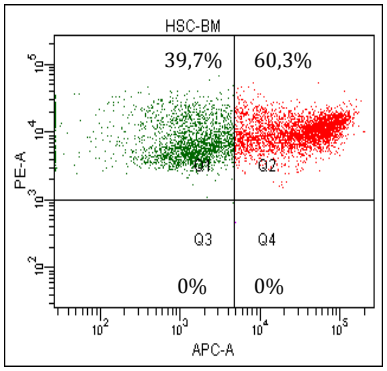
UCB:



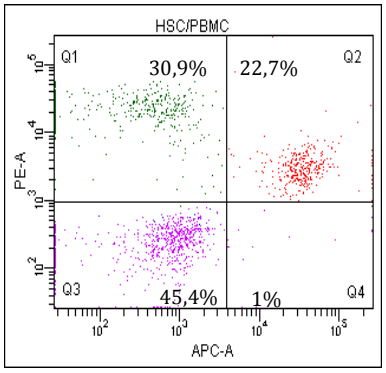


BM





PBMC



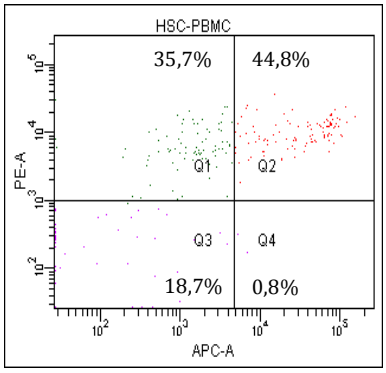
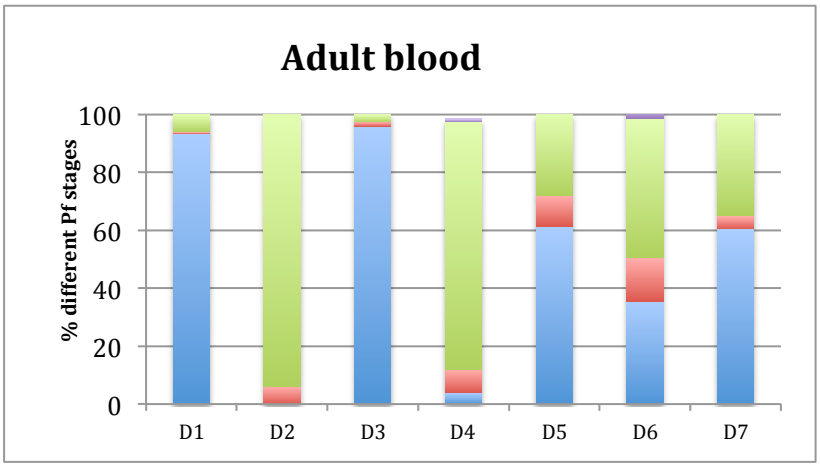
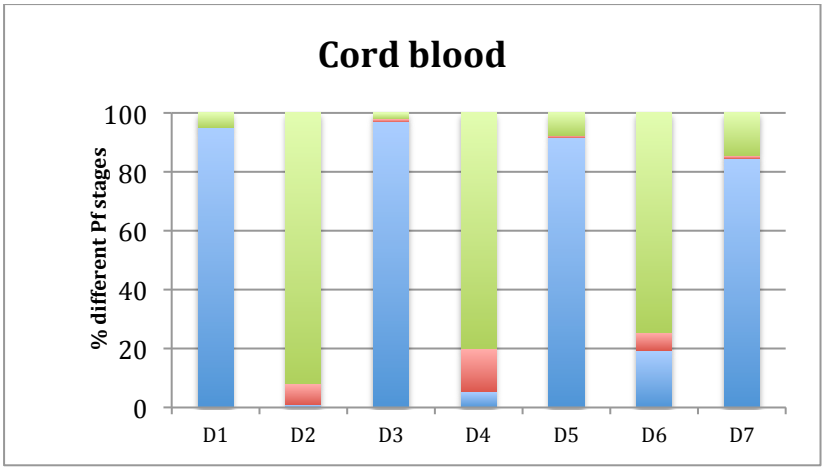


Figure 2: Percentage of each *P. falciparum* stage at different time points of the *in vitro* culture.





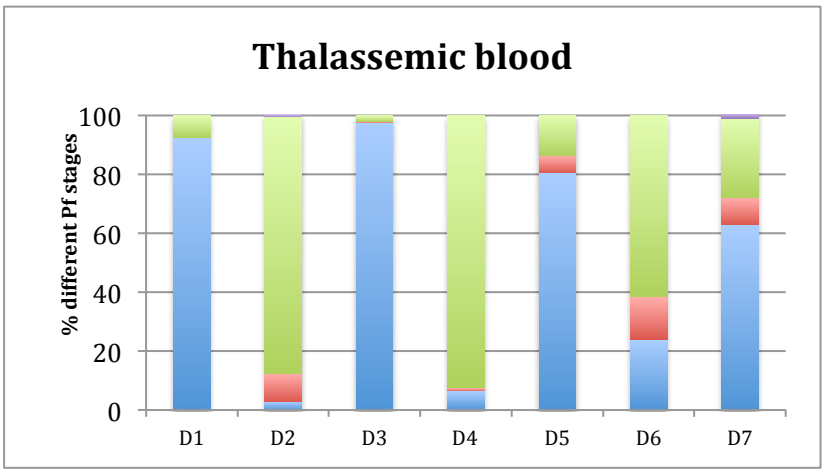


Figure 3: Cytospin of the HSPC-derived reticulocytes 24h after invasion with *P. vivax*. *P. vivax* parasites are shown under arrow.

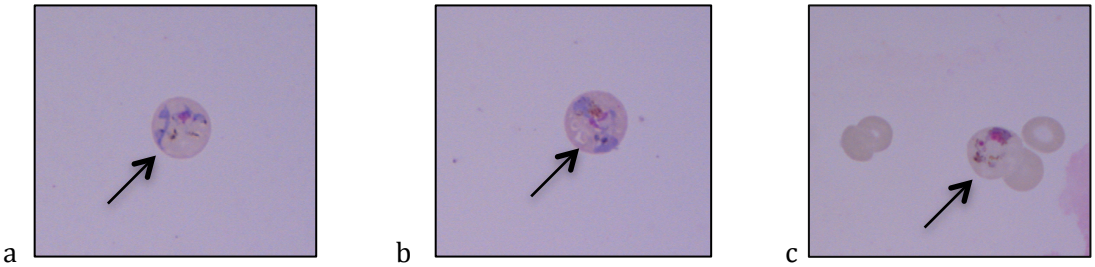
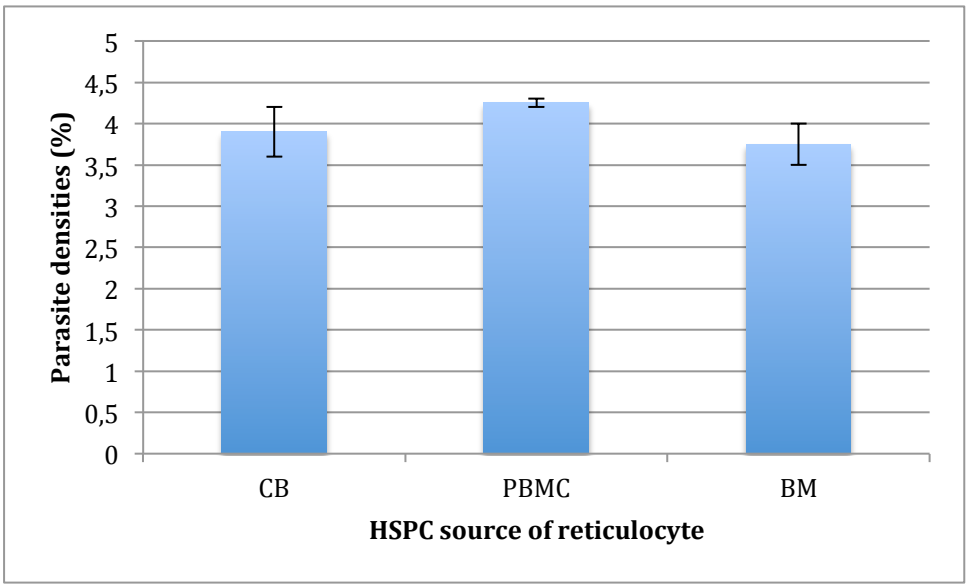


Figure 4: Parasite densities 24 hours post-invasion with *P. vivax*.

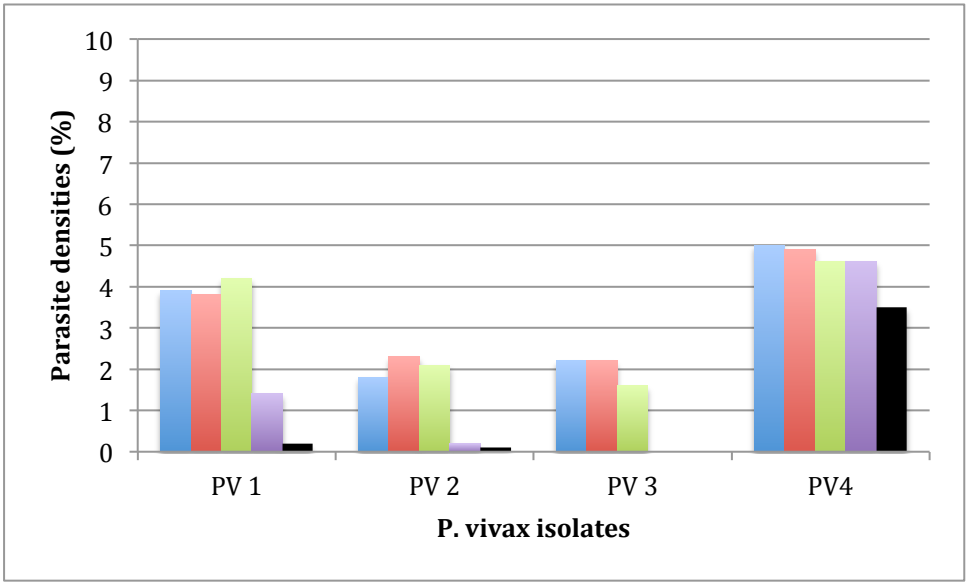
a) Parasite density for different source of reticulocyte with 1 *P. vivax* isolate. The mean and SD of 2 different batches of differentiated reticulocytes was calculated for each source of HSPC and tested for invasion with the same P. vivax isolate.



b) Parasite density for each source of reticulocyte with different *P. vivax* isolates. Parasite densities were counted by dividing the number of *P. vivax* ring-infected cells on

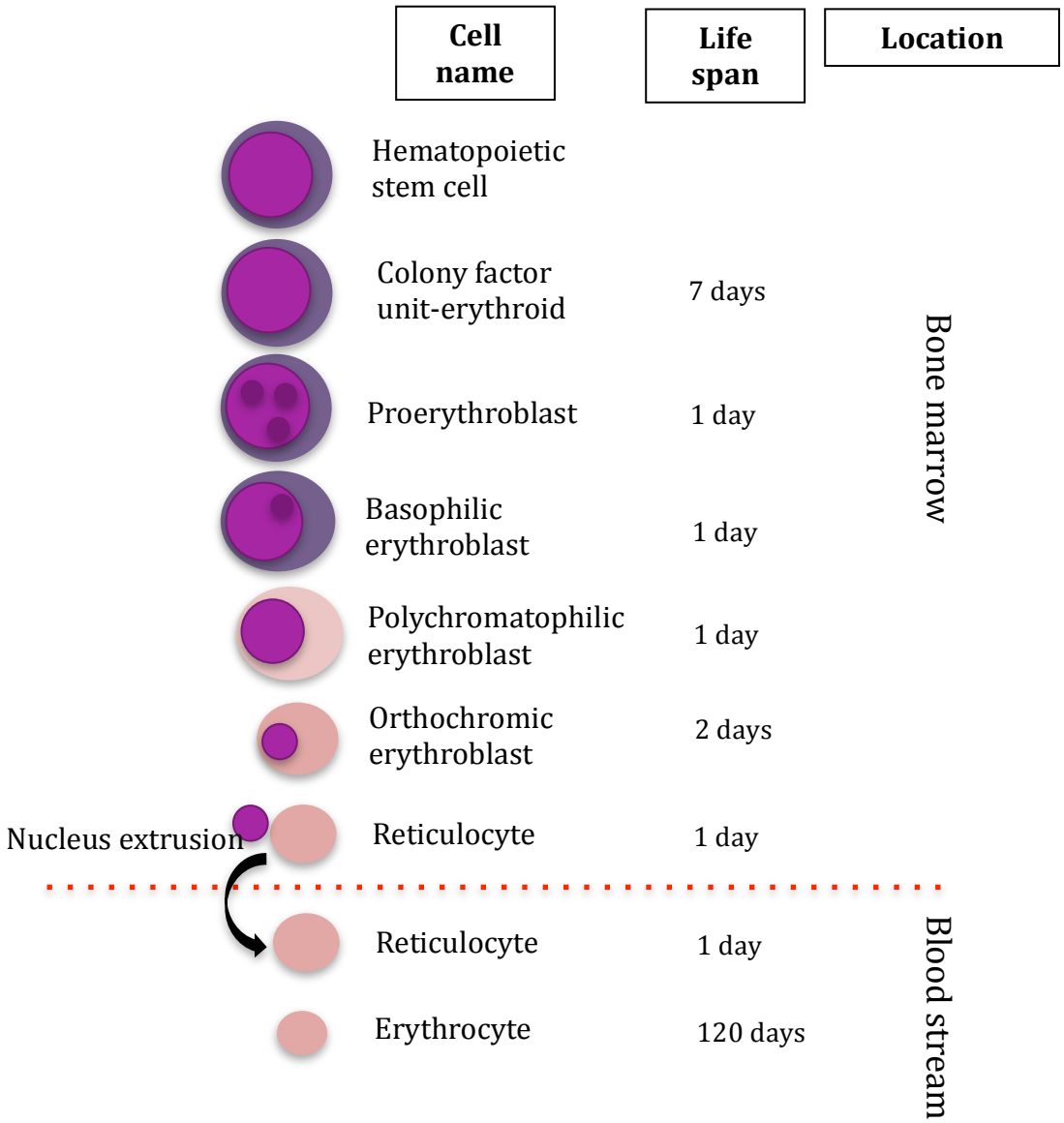
the total number of cells. Different reticulocytes sources were tested; blue: UCB/HSPC-derived reticulocytes, red: BM/HSPC-derived reticulocytes, yellow: PBMC/HSPC-derived reticulocytes, violet: reticulocyte concentrated from UCB, black: reticulocytes concentrated from adult peripheral blood.

PV1 and PV2 were tested with the same batches of HSPC-derived reticulocytes for the 3 different sources (UCB, BM and PBMC). For PV4, the proportion of reticulocyte was 20% for HSCP-derived reticulocytes and respectively 70% and 60% for reticulocytes concentrated from UCB adult peripheral blood



Supplementary data:

Supplementary figure 1: Scheme of the erythropoiesis in vivo. Illustration of the different erythroid cells was drawn with their location and lifespan.

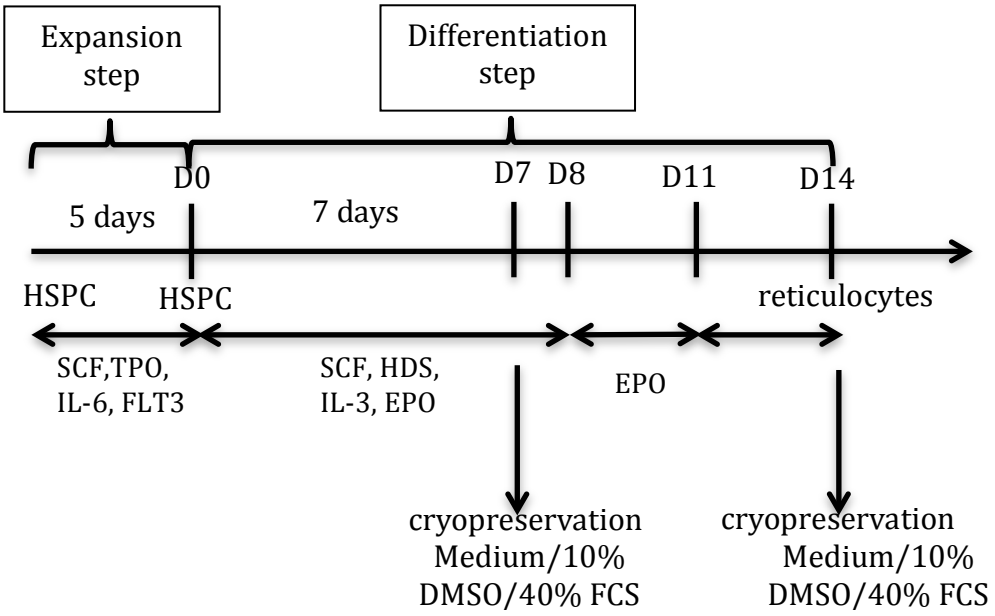

Cell name
Life span
Location
Hematopoietic stem cell
Colony factor unit-erythroid
7 days
Proerythroblast
1 day
Basophilic erythroblast
1 day
Polychromatophilic erythroblast
1 day
Orthochromic erythroblast
2 days
Nucleus extrusion
Reticulocyte
1 day
Reticulocyte
1 day
Erythrocyte
120 days
Bone marrow
Blood stream

Supplementary data 2: Parasite densities 24 hours post invasion for different sources of reticulocytes and different *P. vivax* isolates. Invasion tests were done with HSPC-derived reticulocytes from umbilical cord blood (UCB), bone marrow (BM) and adult peripheral blood mononuclear cells (PBMC) as well as reticulocyte-concentrated blood from umbilical cord blood (UCB cc) and adult peripheral blood (Ad cc). The number of parasitized cells was divided by the number total of cells and multiplied by 100 obtain a percentage of infected cells.

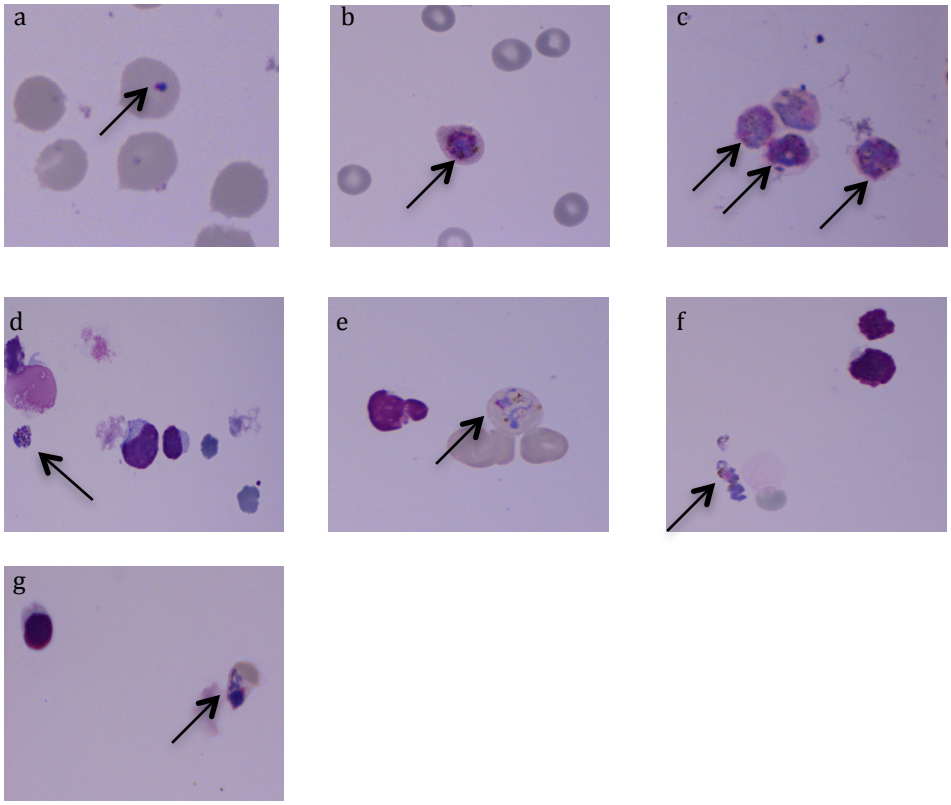
	UCB	BM	PBMC	UCB cc	Ad cc
PV1	3.9%	3.8%	4.2%	1.4%	0.2%
PV2	1.8%	2.3%	2.1%	0.2%	0.1%
PV3	2.2%	2.2%	1.6%	NA	0.2%
PV4	5%	4.9%	4.6%	4.6%	3.5%

NA: Not applicable, no UCB cc was tested for this sample

Supplementary data 3: Timeline of the expansion/differentiation protocol of HSPC into reticulocytes. Timeline showing the different growth factors used during the expansion and differentiation protocols. The days of cryopreservation of the cells were also indicated.



Supplementary data 4: Pictures of the invasion assay protocol for UCB/HSPC source. Cytopsin slides stained with Giemsa a) After P. vivax thawing; b) after 40 hours of culture; c) after percoll concentration; d) starting parasitemia during invasion test with HSPC-derived reticulocytes e) 24 hours post-invasion; f) 48 hours post-invasion; g) 72 hours post-invasion. *P. vivax* parasites are shown under arrow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CHAPTER III: Production of embryonic stem cell-derived-reticulocytes for the *in vitro* culture of *P. vivax*

Production of embryonic stem cell-derived reticulocytes for the in vitro culture of *P. vivax*

Summary:

A continuous *in vitro* culture of *Plasmodium vivax* (*P. vivax*) parasite is an essential tool to understand the biology of this parasite. The preference of *P. vivax* to invade immature erythrocytes (reticulocytes) has been regarded as the main bottleneck for the establishment of an *in vitro* culture system. To date, several protocols have been developed to obtain large amounts of reticulocytes to feed *P. vivax* parasites in culture; including reticulocyte enrichment from different sources of blood [1], and reticulocyte production from hematopoietic stem/ progenitor cells (HSPC)[2].

Here we investigated the use embryonic stem cells (ESC) as an inexhaustible source of cells to generate reticulocytes. Using standard published protocols we here fated ESC to hematopoietic progenitor cells and then reticulocytes. Although some reticulocytes could be generated the efficiency of the culture at generating reticulocytes was low, and insufficient to serve as a source of reticulocytes for *P. vivax* infection.

Introduction:

Plasmodium vivax (*P. vivax)* is now considering as a major health problem due to its morbidity [3] and emergence of drug resistance [4]. The biology of the parasite is still poorly understood as proven by the recent observation of *P. vivax* infections in Duffy-negative populations [5] whereas for a long period, Duffy receptor has been point out to be essential for *P. vivax* entry into erythrocyte [6].

To better understand its biology and the molecular mechanisms involved in parasite invasion process, the development of a continuous in vitro culture is essential. However a major problem to achieve this goal is the preference of *P. vivax for* young erythrocytes (reticulocyte) [7]. Reticulocytes represent a small subpopulation of erythrocytes (0.5-1% of RBC population in adult peripheral blood) with a short lifespan (2 days). If enough reticulocyte cells have to be regularly added in culture to maintain the growth and multiplication of the *P. vivax* parasite, a continuous source of reticulocytes is necessary. Several strategies including reticulocyte concentration from whole blood [8, 9] and reticulocyte differentiation from hematopoietic stem/progenitor cells (HSPC) of different sources have been used, which allow invasion of the parasite but not its long term maintenance *in vitro* [2, 10]. Moreover, even if reticulocytes can be generated from HSPC sources, HSPC do not long-term expand, and hence, variability between donor sources may also interfere with the creation of a reliable *P. vivax* culture system.

Embryonic stem cells (ESC) are pluripotent cells isolated from the inner cell mass of the blastocyst. Compared with adult stem cells, they can proliferate symmetrically without senescence. In addition, the can differentiate into cells of the three germ layers, including hematopoietic cells. Therefore they could provide a consistent source of stem cells for generating reticulocytes. Different protocols of erythrocyte production from human ESC have been recently described [11] opening new opportunities to produce reticulocytes suitable for *P. vivax in vitro* culture establishment.

Here, we attempted to differentiate reticulocytes from ESC to test infection with *P. vivax* using 2 protocols previously published: Lapillonne *et al* [12] who obtained a final amount of 55 to 60% of enucleated erythroid cells and Niwa *et al* [13] who used a serum free medium with a different timeline of growth factor used but obtained a less important amount of enucleated cells (9%).

Material and methods:

i) Inactivated mouse embryonic fibroblast (iMEF) coating

iMEF were plated with a density of 220 000 cells per well in a 6 wells plate and maintained in a iMEF medium with Dulbecco's modified eagle medium high glucose (DMEM HG, Invitrogen), fetal bovine serum (FBS, 15%, HypoClone), penicillin/ streptomycin (0.1X, Invitrogen), L-glutamine (4mM, Gibco), non-essential amino acids (NEAA, 4 mM, Invitrogen)

ii) Maintenance of ESC (H9 cell line)

ESC (H9 cell line) cells were maintained on iMEF in a 6 well plate. They were cultivated in 2.5 mL of ESC medium composed of Dulbecco's modified eagle medium (DMEM) + Hepes (Invitrogen) supplemented with knockout serum replacer (KOS, Invitrogen), L-glutamine (2 mM, Gibco), β-mercapto-ethanol (0.7X, Sigma), NEAA (1X, Invitrogen), penicillin-streptomycin (O.1X, Invitrogen) and basic fibroblast growth factors (b-FGF; 4 10^{-3} µg/mL, R&D system). Cells were placed in a 5% CO_2, 37 °C incubator and the medium was changed every day by removing supernatant and adding 2.5 mL of fresh medium.

Cells were split every 5-7 days depending on how fast colonies expanded. Cells were washed with PBS (Lonza), treated with 1 mL of pre-warmed collagenase IV (0.1%, Sigma) and left 5 minutes at 37°C. After 5 minutes, the supernatant was discarded and 1 mL/ well of ESC medium was added. Using a cell scraper, cells were detached from the bottom of the well and transferred into a 15 mL falcon tube and centrifuged 5 minutes at 0.2 g. The supernatant was discarded and 500 µL of ESC medium added using a 1000 µL tip to get as little colonies as possible. ESC were then platted on a new iMEF layer previously washed with PBS and immerged in 2 mL of ESC medium.

iii) Differentiation protocols

Lapillonne et al protocol: When ESC cultures reached confluence, they were detached using the method described for ESC passaging (see ii).. After the centrifugation step, cells were re-suspended and plated in a low attachment 6 well plate with IMDM medium (Biochrom) supplemented with SCF (100 ng/mL, Bioke), thrombopoietin (TPO, 100 ng/mL, R&D system), FLT-3 ligand (100 ng/mL, R&D system), recombinant human bone morphogenetic protein 4 (BMP-4, 10 ng/mL, R&D system), recombinant human vascular endothelial growth factor (VEGF; 5 ng/mL, R&D system), interleukin-6 (IL-6, 5 ng/mL, R&D system), IL-3 (5 ng/mL, R&D system), EPO (3 U/mL, R&D system) and 5 % human plasma. The cells were cultivated at 5% CO_2 in a 37°C incubator as clumps to form embryoid bodies (EBs, figure 1) and the medium was changed every 2 days by centrifuging EBs 5 minutes at 0.2g. After 20 days of culture, cells were dissociated by incubation 30 minutes at 37°C with collagenase B (0.4 U/mL, Roche applied science) and 10 minutes with dissociation buffer (invitrogen). Dissociated cells were then platted in a 6 wells plate and the hematopoietic stem cells (HSC) protocol previously described applied [14]. The cells were dispended with 4 mL of Iscove's Modified Dulbecco's Medium (IMDM)+ 10% human plasma supplemented with Stem Cell Factor (SCF, 100 ng/ml, Bioke), IL-3 (5 ng/ml, R&D System), Hydrocortisone (HDS, 10-6 M, Sigma), and Erythropoietin (EPO, 3 IU/ml, R&D System). After 8 days, the cells were centrifuged 5 minutes at 0.2 g and resupended in a 25 cm^2 flask with 5 mL of IMDM medium supplemented with EPO (5 IU/mL). After 3 days, the medium was

changed with an IMDM medium without supplementation. The medium was then changed every 3 days.

Niwa et al protocol: 5-6 colonies of ESC were plated in a new 6 wells plate previously coated with 1 mL of matrigel (BD biosciences) with mTeSR1 medium (Stemcell technologies) and left in a 5% CO_2, 37 °C incubator. When the colonies reached nearly 500 µm of diameter, the medium was removed and changed with Stemline II hematopoietic stem cell expansion medium (Sigma) supplemented with insulin-transferrin-selenium-X supplement (ITS, Invitrogen), corresponding to the day 0 of differentiation. For the first 4 days, BMP 4 (20 ng/mL) was added and for the following 2 days, new medium without BMP4 but with VEGF (50 ng/mL) and SCF (10 ng/mL). On day 6 of differentiation, medium was changed to medium supplemented with BMP 4 (20 ng/mL), VEGF (50 ng/mL), TPO (10 ng/mL), IL 3 (50 ng/mL), IL-6 (50 ng/mL, R&D system), FLT-3 ligand (50 ng/mL) and EPO (5 IU/mL). The medium was then changed every 5 days.

iv) FACS and FACS sorting analyses

Briefly, 200 000 cells were centrifuged for 5 min at 650 g. The pellet was re-suspended in 100µL of PBS buffer. 5 µl of CD45-PE (BD bioscience), 10 µL of CD71-APC (BD bioscience), 5 µL of CD36-FITC (BD bioscience), and 5 µL of CD235-PerCP (BD bioscience), were added to the cells and incubated for 15 min at room temperature in

the dark. One tube containing only antibody isotypes coupled to fluorochromes was used as a negative control: 10 µL of IgG1γ-PE (BD bioscience), 5 µL of IgM-FITC (BD bioscience), 5 µL of IgG1 K isotype-PerCP (BD bioscience), and 5 µL of IgG1 K isotype-APC (BD bioscience). After incubation cells were washed with FACS buffer (Miltenyi Biotech) and re-suspended in 500 µL of buffer before analysis. FACS analyses were carried out on a FACScalibur 4 color cytometer (BD bioscience).

Different antibodies were used to characterize the different stages of the cells: CD34-APC (ebioscience), CD45-PE (ebioscience) and CD43-FITC (ebioscience) for hematopoiesis. For erythropoiesis, CD235a-APC (ebioscience), DARC-FITC (BDscience) and CD71-PE (ebioscience) were used. The following isotype controls were used: mouse IgM-FITC, mouse IgG1 K-FITC, mouse IgG1 K-APC, mouse IgG2a-PE and IgG1γ-PE (all from ebioscience).

Cells were centrifuged 5 minutes at 0.2g and resuspended in 100 µL of PBS. For the controls, 5 µL of each isotype control were added to the cells, 5 µL of each antibody was added for the sample tube and no antibody for the bare cell tube. Cells were incubated 15 minutes in the dark before been washed twice with PBS. The pellet was resuspended in 200 µL of PBS before the processing of the tube on a FACS DIVA.

The $CD34^+$ cells, after the 1^{st} step of differentiation, were purified using a FACS ARIA III sorter. based on the expression of $CD34^+$.

v) Magnetic assorted cell sorting (MACS)

The $CD34^+$ population was purified by MACS, cells were washed with PBS and, after a centrifugation of 5 minutes at 0.2 g, resuspended in 500 µL of PBS/0.5% FBS. 100 µL of blocking solution and 100 µL of CD34 magnetics beads (Myltenyl biotech) were added and the tube left 30 minutes at 4°C. After 30 minutes, the cells were washed with the PBS/0.5% FBS solution and passed through a magnet that will retain the cells $CD34^+$ bind to the beads. When all the cells were passed, the column was washed 3 times before to be flushed to recuperate the $CD34^+$ cells.

vi) Ethic statement:

The use of H9 cell line was approved by the ethical commission of the Katholieke Universiteit Leuven.

Results:

i) Production of reticulocytes with the Lapillonne protocol

On day 20, we obtained 3.5 % of CD34 cells and after 43 days, up to 22% of $CD235a^+$ cells. However, very few erythroid cells were enucleated (< 0.01%) (figure 2). A MACS or FACS sorter selection of the $CD34^+$ cells at the end of the 1^{st} step (day 20) did not lead improved enucleation when enriched cells were allowed to differentiate to erythroid cells, using the methods we have used for adult HSPC differentiation.

ii) Production of reticulocyte with the Niwa protocol

After 15 days,, corresponding to the peak of $CD34^+$ cells expression in the work of Niwa *et al*, we could observe 10% $CD34^+$ cells and after 43 days, 7% of the cells were $CD235a^+$(erythroid cells), with very few enucleated cells (< 0.01%). Selection of cells at day 15 based on their $CD34^+$ expression using FACS sorter or MACS did not improve erythroid commitment or enucleation. The $CD34^+$ differentiation protocol was also tested on the $CD34^+$ cells after 15 days but we could not obtain more significant results in term of erythroid differentiation.

Discussion:

Production of erythrocytes from ESC using several different protocols has been described, including co-culture with a human fetal liver cell line [15], co-culture on murine stromal cells (OP9, MS5) [16, 17], erythroid bodies [18], and this for a number of ESC lines [19]., Erythrocytes obtained were reported to resemble erythrocytes found cord blood, with presence of fetal hemoglobin (HbF). In most of these studies the ratio of enucleation was low. Thus, the enucleation process *in vitro* is very challenging [20] an will require optimization to allow the production of erythrocytes.

An additional difficulty to create reticulocytes for *P. vivax* studies is that differentiation should be synchronized to obtain a homogeneous population of erythroblasts that then enucleate simultaneously to become reticulocytes.

Further studies will be needed to develop a consistent and synchronized erythroid-directed differentiation. Finally, the relative high cost of the large number of growth factors needed in important quantities, will likely impede the use of such protocols for *P. vivax* research.

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Fig 1: Embryoid bodies after 5 days of Lapillonne's differentiation protocol

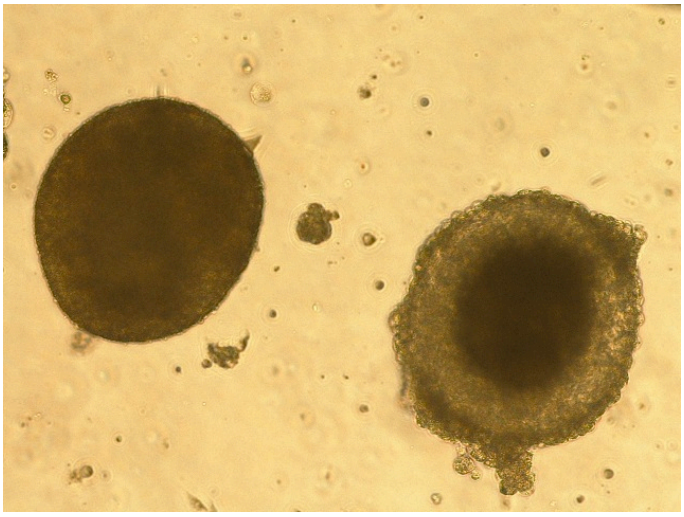
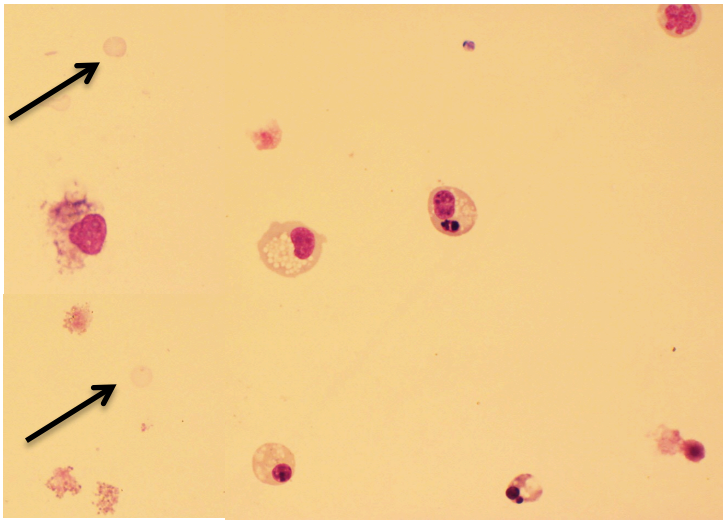


Fig 2: Assemblage of cytospin slides of cells after 43 days in culture. Enucleated cells are shown under arrows.



DISCUSSION

The establishment of a reliable and continuous *in vitro P. vivax* culture is needed to better understand its biology and the molecular mechanisms involved in the process of reticulocyte invasion [91]. The recent reports of *P. vivax* infections in Duffy negative-individuals [92] demonstrate that the mechanisms of invading reticulocytes and other aspects of the biology of the parasite are largely unknown. Despite the several attempts over the last 100 years, a satisfactory and reproducible model supporting the whole blood cycle of *P. vivax* is not currently available [40, 42, 93]. A critical factor for achieving a successful *in vitro P. vivax* culture is the access to a continuous source of reticulocytes. During my PhD thesis, we assessed the suitability of different sources of stem cells to obtain reticulocytes that can be infected with *P. vivax*, and wished to develop a model that would allow *P. vivax* to multiply and mature to every schizogony stage.

Use of HSPC as source of reticulocytes

- HSPC isolated from umbilical cord blood

Reticulocytes derived from $CD34^+$ cells isolated from umbilical cord blood could provide a good source of reticulocyte for *P. vivax* as UCB is considered medical waste and is easy to obtain. Although UCB derived CD34+ cells had already

been used for culturing *P. vivax* [54], the percentage of reticulocytes obtained was very low (0.5% after 14 days of culture compared to 0.5-1% in vivo) and did not represent a significant advantage compared to peripheral blood. Conversely, we were able to increase the percentage of reticulocytes obtained after 14 days of culture from HSPC-UCB source to 20% and these could be successfully used to culture *P. falciparum* (for at least 15 days) and could be invaded by *P. vivax* (up to 2.5 % of parasitemia) though no parasite multiplication could be observed. The invasion rate was dependent of the *P. vivax* isolate, but not on the UCB/HSPC derived reticulocyte batches, indicating that the latter could be used for the culture. In addition, invasion was not affected by cryopreservation so that it would be possible to start a *P. vivax* culture by using cryopreserved cells, both parasite isolates and $CD34^+$-derived reticulocytes, opening the possibility of carrying out *ex vivo* invasion assays in laboratories localized in non-endemic countries.

- HSPC isolated from different sources

To identify the best source of $CD34^+$ cells to obtain reticulocytes suitable for *P. vivax* invasion, we tested $CD34^+$ enriched cell populations from un-mobilized peripheral blood (PB), bone marrow (BM) and umbilical cord blood (UCB). Reticulocytes produced from $CD34^+$ cells isolated from UCB [53] BM and PB [94] were shown to be permissive to *P. vivax* though it is unknown if there is any difference in suitability as a direct comparison between sources has never been done.

We could differentiate $CD34^+$ enriched fractions from different sources and characterize the reticulocytes obtained. The reticulocyte production after 14

days from UCB-CD34+ enriched cells reached 18 %. The reticulocytes contained HbF and the Hb concentration was higher than the *in vivo* range while reticulocytes from CD34+ enriched BM and PB contained HbA, with concentrations higher than the normal range, but the percentage of reticulocytes obtained after 14 days was significantly higher than for UCB/CD34+ cells.

We also added an expansion step prior to differentiating of the CD34+ enriched UCB, BM and PB, with the hypothesis that if more CD34+ cells were present at the beginning of reticulocyte production, a significantly larger numbers of reticulocytes could be generated from a single sample. CD34+ enriched cells isolated from UCB expanded significantly more than those from BM or PB, consistent with published studies and with the concept that UCB/CD34+ have greater proliferative potential [95]. Combining the expansion as well as the enucleation capacity, UCB/CD34+ cells appear to be the most appropriate source to generate reticulocytes in suitable quantities to provide target cells to *P. vivax in vitro*.

The reticulocytes produced from the 3 different HSPC sources (with or without expansion step) could be invaded and allowed the intra-erythrocyte development of *P. falciparum* for at least 1 week. No differences were seen between the cell sources in terms of permissiveness and parasite growth. These reticulocytes could also be invaded by *P. vivax* with the same efficiency; however few new ring forms could be observed after 3 days of culture, regardless of the HSC sources, and the parasites could not develop further.

Interestingly, while comparing the *P. vivax* invasion rate of differences sources of reticulocytes (CD34+-derived or blood-concentrated) by *P. vivax*, we observed that permissiveness of the CD34+-derived reticulocytes was higher than that of reticulocyte-concentrated UCB or from adult peripheral blood.

These results appear consistent with the studies by Paterakis *et al* in 1993 [96] in which the reticulocyte populations in UCB and PB were compared. They classified reticulocytes in 3 groups based on RNA content: young, average and old. They found 13% of young reticulocyte in UCB while only 1% could be found in PB. This may explain the differences of invasion observed in the $CD34^+$-derived reticulocytes, which are harvested at the peak of enucleation, and hence likely to contain a larger fraction of "young" reticulocytes compared with reticulocytes concentrated from UCB and PB.

These observations are also in line with the study of Martin-Jaular *et al* [97]who demonstrated a preference of *Plasmodium yoelii* (a mouse-infecting plasmodium that has also a preference for reticulocyte as target) for $CD71^{high}$ expression cells (immature reticulocytes).

If these results are confirmed, this might provide a good model to study the mechanisms underlying *P. vivax* invasion.

Influence of hemoglobin on *Plasmodium* development

It has previously been hypothesized that using UCB/$CD34^+$ cells as source for reticulocytes, which contain HbF, would be less suitable for the intra-erythrocyte development of *P. vivax*. Pasvol et al [68] described a slowdown of the intra-erythrocyte development of *P. falciparum* but for *P. vivax* no data exists. In order to revise and test this hypothesis, we investigated the influence of HbA and HbF on the intra-erythrocyte development of both *P. falciparum* and *P. vivax*. We also investigated the importance of the hemoglobin concentration on the parasite culture by testing minor β thalassemia blood whose RBC have less hemoglobin than normal.

None of the conditions tested, i.e. HbF and minor β thalassemia, was associated with a significant delay in the *in vitro* maturation of *P. falciparum* whose development remained synchronous for at least 7 days. These results are opposite to those published by Pasvol *et al* and could be explained by the difference of gas mixture used to cultivate *P. falciparum* under hypoxia conditions (5% O_2). Therefore, the greater affinity of HbF for O_2 [98] may compensate for the hypoxic condition and thus decrease the influence of HbF on the parasite development.

HbF did not influence the invasion rate of $CD34^+$-derived reticulocytes from UCB (HbF) vs BM and PB (HbA) by *P. vivax* either. However, irrespective of the cell source, parasites could not complete a first schizogony cycle *in vitro,* and we could only detect rare parasites having survived for 3 days. Though from these results it is not possible to identify the important factors influencing the intra-reticulocyte development (or the lack of it) of *P. vivax in vitro*, probably HbF is not one of them.

ESC- derived reticulocytes

Whereas $CD34^+$ cells are a good source of cells to generate reticulocytes for *P. vivax* culture, the number of cells that can be produced remains limited, even when performing $CD34^+$ cell expansion. Although we did not see significant differences between different UCB donors as it relates to *P. vivax* invasion, inter-individual variations might still pose a problem for the reticulocyte generation. Therefore, the use of human embryonic stem cells that have theoretically limitless expansion potential could be an alternative source of cells to generate

reticulocytes. ESCs are amenable to genome editing that would result in a modification of cell surface receptors or intracellular proteins, possibly opening the opportunity for not only identifying the receptors involved in the invasion but also for recognizing essential factors needed for the development of *P. vivax* development *in vitro*. To date, several protocols exist to produce erythrocytes from embryonic stem cells (ESC) [99-101] or induced pluripotent stem cells (iPSC) [102-104].

One of the main difficulties met for the use of ESC in the production of reticulocytes/ erythrocytes is the enucleation step that is difficult to reproduce efficiently *in vitro* [105].

Concerning the generation of reticulocytes, the differentiation should be stopped in the extremely narrow time window of 48 hours, between the orthonormoblast stage and erythrocyte stage. To date, most of the ESC differentiation protocols aim to generate erythrocytes and thus are not concerned by this time window problem.

Nevertheless, different ESC differentiation protocols are available with different approaches [103] allowing to choose the more suitable one.

The now well-established differentiation of $CD34^+$ into reticulocytes can also be a great asset as we could generate $CD34^+$ cells from ESC and then apply the $CD34^+$ differentiation protocol. As the $CD34^+$-derived reticulocytes have shown their permissiveness to *P. vivax* and seemed to be the most suitable source of reticulocyte for the parasite, research on ESC-derived reticulocytes should be consider a priority for the generation of reticulocytes for a *P. vivax in vitro* culture.

Future perspectives

During my thesis, we focused on the reticulocyte production in order to provide cell targets to sustain *P. vivax* development *in vitro*. Although we made progress in finding cell populations that can produce a large numbers of reticulocytes that can also be cryopreserved and later invaded by *P. vivax*, the parasite could not be maintained in culture for more than one schizogony cycle. We also demonstrated that HbF did not influence the development of *P. falciparum* or the invasion of *P. vivax*. The 1st schizogony of *P. vivax* was also possible for rare parasites and we could hypothesis that HbF would not have such an important influence on the intra-reticulocyte development of *P. vivax*.
Future studies should focus on differences between infection occurring *in vivo* vs. *in vitro* to identify the essential proteins or pathways that may explain the inability of *P. vivax* to develop further *in vitro*. Metabolomics studies could be used to identify those factors as previously shown for *P. falciparum* [106]. If such differences could be identified, they could be tested to improve the *in vitro* conditions of the *P. vivax* culture.

The preliminary observations on the preference of *P. vivax* to invade immature reticulocytes also need to be confirmed. For this, a comparison of *P. vivax* invasion rates between reticulocytes harvested after 14, 15 and 16 days of differentiation would be useful. The level expression of CD71 will be monitored by FACS to discriminate the immature from the old reticulocytes.

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Aknowledgment

To those who supported me and those who did not.

Curriculum Vitae

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WORK EXPERIENCE

2010-Now: Institute of Tropical Medicine, Antwerp (Belgium)

Topic: **Culture of *Plasmodium Vivax***

→ Production of reticulocytes from Hematopoietic Stem Cells, *P. vivax* invasion tests

2009: 6 month as Visiting Scientist at the New York Blood Center (Lindsay F Kimball Research Institute) in collaboration with the Evandro Chagas Institute in Belem (Brazil)

Topic: **Invasion Profile of *Plasmodium falciparum* field Isolates in Red Blood Cells.**

→ Project management, coordination between several teams, training of a master student and local team to biological techniques, *Pf* culture, invasion tests, RBC enzyme treatments

2006-2008: Research assistant (international volunteer for French cooperation) at the "Centre International de Recherches Médicales de Franceville (CIRMF)" Gabon (Central Africa).

Topic: **Analysis of immune response against putatives apoptogenic recombinant antigens of *P. Falciparum***

→ Project management, Student training, stock management, biological technical advises to center researcher

2005: 6 month (master thesis) at the « Commissariat à l'Energie Atomique (CEA)/ Inserm U566 » Fontenay aux Roses

Topic: **Oocyte maturation in vitro and apoptotic modelling.**

→ Work on mice oocytes, Oocyte maturation from Mouse ovarian follicles, confocal and electronic microscopy

2004: 2 month (1st year master) at the "Centre de Biophysique Moléculaire" (CNRS, Orléans)

Topic: **Directed mutagenesis of Human Phosphatidylethanolamine Biding Protein (PEBP).**

EDUCATION

2005: Master in Bio industrials techniques (Orléans University, France)

Year 1:

- Molecular biology
- Genetic
- Neuroscience
- Animal physiology
- Physiopathology

Year 2:

- Cell culture
- Immunology
- Instrumentation
- Bio imagery
- Gene therapy

2003: Bachelor's Degree of Cellular Biology and Physiology (Orléans University, France)

SCIENTIFIC ACHIEVEMENTS

Peer-reviewed journal publications:

Florian Noulin, Javed Karim Manesia, Anna Rosanas-Urgell, Annette Erhart, Céline Borlon, Jan Van Den Abbeele, Umberto d'Alessandro, Catherine M. Verfaillie. Influence of CD34+ source and hemoglobin type for culturing *Plasmodium* parasites. Under review

Noulin F, Borlon C, Van Den Abbeele J, D'Alessandro U, Erhart A. *1912-2012: A century of research on Plasmodium Vivax in-vitro culture.* Trends Parasitol. 2013 Apr 24. pii: S1471-4922(13)00056-1. doi: 10.1016/j.pt.2013.03.012.

Noulin F, Borlon C, van den Eede P, Boel L, Verfaillie CM, et al. (2012) Cryopreserved Reticulocytes Derived from Hematopoietic Stem Cells Can Be Invaded by Cryopreserved Plasmodium vivax Isolates. PloS one 7: e40798.

Ulrick Bisvigou ESZ-E, **Florian Noulin**, Rafika Zatra, Ludovic Mevono, Jean-Bernard Lékana-Douki, Dominique Mazier, Frédérick Gay and Fousseyni S. Touré Ndouo (2012) Antigenicity of Synthetic Peptides Derived from Plasmodium Apoptosis-Linked Pathogenicity Factors. Journal of Life Sciences 6: 587-594.

Ouwe-Missi-Oukem-Boyer O, Toure Ndouo FS, Ollomo B, Mezui-Me-Ndong J, **Noulin F**, Lachard I, Ndong Atome G.R, Makuwa M, Roques P, Branger M, Preux PM, Mazier D, Bisser S. *Hepatitis C Virus Infection May Lead to Slower Emergence of P. falciparum in Blood.* PLoS One. 2011 Jan 10;**6**(1)

Conference presentations:

Mai 2013: Poster presentation at the vivax conference in Barcelona: "Influence of Hematopoietic stem cell (HSC) source to produce reticulocytes for the *in vitro* culture of *Plasmodium vivax*"

August 2012: Poster presentation at the 12th ISEH meeting (Amsterdam): "Reticulocytes derived from hematopoietic stem cells can be successfully cryopreserved and later used for P. vivax invasion tests"

December 2011: Poster presentation at the 60th ASTMH meeting (Philadelphia): "Reticulocytes derived from hematopoietic stem cells can be successfully cryopreserved and later used for P. vivax invasion tests"